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Acylation of *Escherichia coli* Hemolysin: A Unique Protein Lipidation Mechanism Underlying Toxin Function

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INTRODUCTION

Protein toxins are prominent virulence factors of many pathogenic bacteria. While toxins of gram-positive bacteria do not generally require activation, many toxins of gram-negative bacteria are translated in an inactive form and require a processing step, most often a proteolytic cleavage, to generate the active form. Enzymatic toxins such as Shiga toxin, cholera toxin, pertussis toxin, diphtheria toxin, and *Pseudomonas aeruginosa* exotoxin A undergo proteolytic cleavage to produce a catalytic A fragment acting in the eukaryotic target cell (279). Similarly, many nonenzymatic toxins that insert into eukaryotic membranes require proteolytic cleavage to allow oligomerization and pore formation; e.g., *Vibrio cholerae* El Tor hemolysin is cleaved at its N terminus, while *Aeromonas* aerolysin, *Clostridium septicum* alpha-toxin, and *P. aeruginosa* cytotoxin are cleaved at their C termini (13, 219, 236). The pore-forming hemolysin (HlyA) of *Escherichia coli* represents a unique class of bacterial toxins that require a posttranslational modification for activity, specifically the covalent amide linkage of fatty acids to internal lysine residues; the suggestion that activation of the *Serratia* and *Proteus* hemolysins ShlA and HpmA involves covalent modification has not been supported by subsequent experiments (123). After introducing the hemolysin toxin, this review will focus on the posttranslational mechanism of HlyA modification (maturation) and will examine its relationship to protein modifications involving lipid groups that occur in prokaryotes, lower eukaryotes, and mammalian systems. The functional significance of protein lipidation in these examples will then be reviewed and used as a basis for discussion of the possible role(s) of acylation in the cytokine-inducing and pore-forming functions of the HlyA toxin.

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TABLE 1. The toxins of the RTX exoprotein family

Bacterium	Toxin	Size (kDa)	HlyA identity (%)
E. coli	HlyA	110	
	EhxA	107	61
	EaggA[a]	120[b]	?
P. vulgaris	PvxA	110[b]	73[c]
M. morganii	MmxA	110[b]	60[c]
A. pleuropneumoniae	ApxIA	110	59
	ApxIIA	102	49
	ApxIIIA	113	56
A. actinomycetemcomitans	AaltA	114	54
A. suis	AshA	102	49
P. haemolytica	LktA	102	49
P. haemolytica-like	PllktA	102	48
B. pertussis	CyaA	178	31[d]
M. bovis	MbxA[a]	110[b]	?

[a] No gene yet identified.
[b] Sodium dodecyl sulfate-polyacrylamide gel electrophoresis.
[c] Partial sequence comparison.
[d] Comparison with N-terminal adenylate cyclase-deleted CyaA.

E. coli HlyA and a Family of Bacterial Pore-Forming Toxins

HlyA is an important virulence factor in *E. coli* extraintestinal infections such as those of the upper urinary tract (81, 107, 124, 318). It is one of a close family of membrane-targeted toxins assumed or proven to be influential not only in urinary tract infections but also hemorrhagic intestinal disease, juvenile periodontitis, pneumonia, whooping cough, and wound infections; these toxins include enterohemorrhagic O157 *E. coli* hemolysin (257), the leukotoxin of *Pasteurella haemolytica* (291), the hemolysins and leukotoxins of *Actinobacillus* spp. (46, 52, 89, 173), the bifunctional adenylate cyclase-hemolysin of *Bordetella pertussis* (100), and the hemolysins of *Proteus vulgaris* (167, 319), *Morganella morganii* (167), and *Moraxella bovis* (101) (Table 1). These toxins have 30 to 75% sequence identity to *E. coli* HlyA and share (i) posttranslational maturation, (ii) a C-terminal calcium-binding domain of acidic glycine-rich nonapeptide repeats that has led to the RTX (repeat toxin) family nomenclature, and (iii) export out of the cell by type I secretion systems (34, 59, 129, 171, 320). The posttranslational modification is unique to this toxin family, but Ca^{2+} binding and type I secretion are both common to other bacterial proteins (18, 34, 86, 221, 301). In HlyA there are between 11 and 17 glycine-rich repeats. When Ca^{2+} is bound (one calcium ion per repeat), these form short β-strands organized in an unusual "spring-like" structure called a parallel β-barrel or β-superhelix (18). Calcium binding is an absolute requirement for cytotoxic activity (36, 37, 187, 232) and occurs outside bacteria following export, the intracellular level of free calcium in *E. coli* being very tightly regulated to 0.1 μM (92), a level too low for HlyA activity.

Synthesis and Export of HlyA Toxin

The synthesis, maturation, and secretion of *E. coli* HlyA are determined by the *hlyCABD* operon (81, 132, 171, 225) (Fig. 1). The membrane-located export proteins are synthesized at a lower level than the cytosolic HlyC and pro-HlyA, in part due to transcription termination within the *hlyCABD* operon (81). This termination is suppressed by the elongation protein RfaH and a short 5′ DNA sequence, *ops* (operon polarity suppressor) (7, 8, 60, 225, 311), that act together to allow the transcription of long operons such as *hly*, *rfa*, and *tra* that encode the synthesis and export of extracellular components important to the virulence and fertility of gram-negative bacteria (7, 9).

The pro-HlyA protoxin is matured in the cytosol to the active form by HlyC-directed fatty acylation (see below). The maturation increases the hydrophobicity of the protein but is not required for export (186). *E. coli* HlyA and its toxin relatives are all secreted across both membranes by the type I export process employing an uncleaved C-terminal recognition signal (223, 281) but no N-terminal leader peptide (82) or periplasmic intermediate (83, 168). The HlyA secretory apparatus comprises HlyB (an inner membrane traffic ATPase), HlyD (an inner membrane protein that is suggested but not shown to bridge to the outer membrane), and TolC (an outer membrane protein) (260, 313, 315). In *E. coli* and most other pathogens, TolC is encoded by a gene separated from *hlyCABD*, but in *B. pertussis* the toxin locus includes *tolC* (*cyaE*). Type I secretion signal sequences have been located within the C-terminal 24 to 80 amino acids (137, 168, 293). The HlyA C terminus is predicted to contain an amphipathic helix (159, 281), and circular dichroism and nuclear magnetic resonance spectroscopy of the HlyA signal has shown that α-helices are formed in a membrane mimetic environment (330). Despite a lack of identity between their primary sequences, the interchangeability of the export genes suggests that higher-order structures in the signals are shared among the extended family of hemolysins, leukotoxins, and proteases (256, 262, 301, 329).

HlyB has an integral membrane domain fused to an ATP-binding cassette (traffic ATPase) cytoplasmic domain, which undergoes conformational change on ATP binding (170) and couples ATP hydrolysis to HlyA export (166). Topological models proposed from fusion data suggest that HlyB inserts in the cytoplasmic membrane via six helical transmembrane segments with both its N and C termini in the cytoplasm. The predicted HlyB cytoplasmic loops are large and positively charged, while the periplasmic loops are small (97). The function of HlyD is less clear, but it is proposed to have a single transmembrane segment with the N terminus in the cytoplasm and a large C-terminal region in the periplasm (260). TolC is a minor outer membrane protein believed to form ion-permeable channels. Electron microscopy of two-dimensional lattices of TolC in phospholipid bilayers has revealed it to be a trimeric porin-like structure with a C-terminal extramembrane domain believed to form a periplasmic bridge to the energized inner membrane components of the translocation complex (172). HlyBD-dependent type I secretion shares with the Sec secretion across the cytoplasmic membrane an early requirement for the total proton motive force (PMF) but also has a late stage that does not require PMF, membrane potential, or the proton gradient. A translocation intermediate identified in the PMF-independent late stage is closely associated with the inner membrane, possibly in a translocation complex spanning both membranes (169). The belief that HlyA interacts with HlyB is compatible with the finding that suppressor mutations in HlyB partially compensate for mutations in the HlyA secretion signal (273), and a view of the assembly of a transport-competent complex occurring in an ordered manner is now being substantiated by cross-linking experiments (34, 300). A consequence for HlyA secretion of the virtual absence of Ca^{2+} ions in the bacterial cytoplasm is that the glycine-repeat struc-

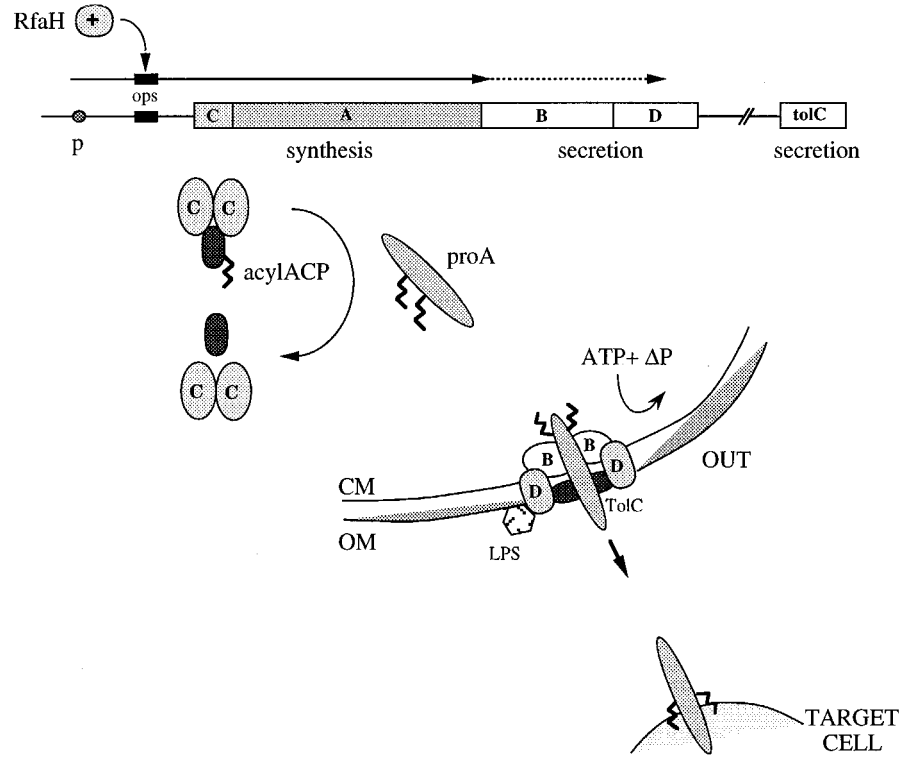

FIG. 1. Hemolysin synthesis, maturation, and export by *E. coli*. (CM, cytoplasmic membrane; OM, outer membrane; ΔP, total PMF). Expression of the hemolysin operon is governed by components upstream of the *hly* genes. The *hlyA* gene encodes inactive prohemolysin, which is activated by HlyC. HlyA is secreted by a type I process. Ca^{2+} binds to the glycine-rich repeats of the toxin externally before interacting with the mammalian membrane.

tures should be flexible, allowing translocation of the polypeptide across the bacterial membranes in an unfolded state.

BIOLOGICAL EFFECTS OF HlyA AND RELATED TOXINS

Host Cell Specificities of the Toxin Family

The toxins of the HlyA (RTX) family have been grouped on the basis of their lytic and toxic effects on mammalian host cells: hemolysins exhibit little target cell specificity, while leukotoxins have pronounced species- or cell-specific effects (59, 320) (Tables 2 and 3). *E. coli* HlyA has a wide spectrum of cytocidal activity, attacking erythrocytes, granulocytes (28, 91), monocytes (29), endothelial cells (294), and renal epithelial cells of mice, ruminants, and primates (155, 212). The 61% identical hemolysin (Ehx) from enterohemorrhagic *E. coli* O157 lyses sheep and human erythrocytes and kills bovine leukocytes (albeit with a specific activity more than 20-fold lower than that of HlyA), but, unlike HlyA, it has virtually no activity against human leukocytes (16). These contrast with the *P. haemolytica* and *Actinobacillus actinomycetemcomitans* leukotoxins which have little erythrolytic activity but a potent cytotoxic activity toward phagocytic cells of particular animals: the *P. haemolytica* leukotoxin (LktA) lyses leukocytes only from cattle, sheep, and other ruminants (274), while the *A. actinomycetemcomitans* leukotoxin (AaltA) specifically lyses human and primate polymorphonuclear lymphocytes (328). *A. pleuropneumoniae* produces hemolysins (ApxIA and ApxIIA) and a leukotoxin (ApxIIIA) (89) (Table 2).

How such reported host cell specificities arose during the evolution of the toxin family is not clear from phylogenetic trees derived from DNA sequences (320), and the physical basis for cell specificity is unresolved. Studies performed on hybrid toxins created by the exchange of putative domains between HlyA, LktA, ApxIIA, and AaltA have indicated that specificity is unlikely to lie in a single discrete feature. Activity against erythrocytes has been correlated with a central region of HlyA and an N-terminal region of ApxIIA (88). The ability to kill leukocytes has been mapped to C-terminal regions of LktA and ApxIIA but appears less sharply defined in HlyA (88, 202). The critical region of AaltA required for recognition of human target cells spans its glycine-rich repeats (175), whereas the specificity of LktA for ruminants has been linked to a feature at its N terminus (88).

Ambiguity in the experimental definition of the target cell range may contribute to the lack of understanding of targeting by these toxins. Targeting has been defined by the cytotoxic end result (death), but this is the result of a multistage mechanism that might involve receptor recognition, membrane insertion, and protein-protein interaction. There are therefore many reasons why a particular cell line might be insensitive to a particular toxin. The toxin may not interact with the membrane, and this may occur for AaltA with human erythrocytes (298) and for LktA with nonbovine erythrocytes (15, 42). The toxin may adsorb to the membrane but fail to insert into the lipid bilayer, and this may be the behavior of inactive, nonacylated protoxins. Even if the toxin does permeabilize the membrane, the cell line will appear insensitive if it possesses a lesion repair mechanism. It is also possible that the bacterial host will affect the final toxin activity. The *B. pertussis* CyaA hemolysin synthesized in *E. coli* has a fourfold-lower hemolytic activity than does the native CyaA (109), and while ApxIIA protein secreted from its native host *A. pleuropneumoniae* is both hemolytic and cytotoxic, expression of the *apxIICA* genes in *E.*

TABLE 2. Mammalian host cells known to be targeted by the hemolysins and leukotoxins of the HlyA family

Toxin	Target species	Target cells	Reported effects
HlyA	Human, monkey, pig, rat, mouse, horse, sheep, cow	Erythrocytes, leukocytes, lymphocytes, epithelia, endothelia	Cytolytic, cytotoxic, NO production, cytokine production, cytoskeletal rearrangement, superoxide production, leukotriene production, receptor shedding
EhxA	Human, sheep, cow	Erythrocytes, leukocytes	Cytolytic, cytotoxic
ApxIA/ApxIIA	Pig, cow, rabbit	Erythrocytes, leukocytes, lymphocytes, macrophages, endothelia	Cytolytic, cytotoxic, superoxide production
ApxIIIA	Pig	Leukocytes, macrophages	Cytotoxic
AaltA	Human, ape, monkey	Leukocytes, lymphocytes	Cytotoxic
AshA	Pig, horse, sheep, cow	Erythrocytes	Cytolytic
LktA	Sheep, goat, cow	Leukocytes, lymphocytes, macrophages, platelets	Cytotoxic, superoxide production, leukotriene release, cytokine production, cytoskeletal rearrangement
PllktA	Pig, cow	Leukocytes, lymphocytes	Cytotoxic
CyaA	Human, sheep	Erythrocytes, leukocytes, lymphocytes, macrophages	cAMP production, cytolytic, cytotoxic, inhibits superoxide production
MbxA	Sheep, cow	Erythrocytes, lymphocytes, epithelia	Cytolytic, cytotoxic

coli generates a toxin that is cytotoxic but has little or no hemolytic activity (305). LktA from *E. coli* carrying the *P. haemolytica lktCA* genes caused weak lysis of erythrocytes despite reports that LktA has a target cell range limited to leukocytes and platelets (87, 125). Continued biochemical investigation of the action of these toxins should establish whether continued division into hemolysins and leukotoxins is appropriate.

An explanation for the apparent cell specificity of these toxins might be offered by their recognition of specific membrane receptors. The existence of receptors for many bacterial toxins, both enzymatic and pore forming, is well established. Diphtheria toxin enters mammalian cells by using the epidermal growth factor-like growth factor as a receptor (220), and the AB_5-type toxins such as cholera toxin, Shiga toxin, and verotoxin bind to cell surface ganglioside lipids (279). The binding of *Staphylococcus aureus* pore-forming leukotoxins to human polymorphonuclear neutrophils is initiated via a calcium channel or a receptor linked to a calcium channel (280). Aerolysin of *Aeromonas hydrophila* binds to erythrocytes and T lymphocytes via high-affinity receptors that have been shown to be glycoproteins with common glycosylphosphatidylinositol (GPI) anchors (73); *Clostridium perfringens* enterotoxin also uses several structurally related proteins as receptors (152); and the receptor for *C. difficile* toxin A has been identified as sucrase-isomaltase (245). Whether there is a receptor for the *E. coli* hemolysin is uncertain. Dose-response binding assays performed by one group support an upper limit of 4,000 HlyA binding sites per erythrocyte, implying at least some degree of specificity (15). However, this contrasts with results from others, who estimated up to 50,000 toxin molecules bound per erythrocyte and, using radiolabelled hemolytically active HlyA, subsequently found no evidence for saturation in binding to erythrocytes and leukocytes, indicating that this process is not

TABLE 3. Division of toxins into hemolysins and leukotoxins in relation to the amino acids at the conserved KI and KII positions of HlyA

Toxin	Activity	Position of:		Total no. of amino acids
		KI	KII	
HlyA	Hemolysin	K564[a]	K690[a]	1,024
EhxA	Enterohemorrhagic hemolysin	K550	K675	998
ApxIA	Hemolysin	K560	K686	1,022
ApxIIA	Hemolysin	K557	N687	956
ApxIIIA	Leukotoxin	K571	K702	1,049
AaltA	Leukotoxin	K562	K687	1,050
AshA	Hemolysin	K557	N687	956
LktA	Leukotoxin	K554	N684	953
PllktA	Leukotoxin	K550	S680	947
CyaA	Adenylate cyclase/hemolysin	K860	K983[a]	1,706

[a] Identified as acylated residue in in vivo-expressed toxin.

conditional on specific receptors (31, 76). The absence of a specific receptor could be the reason why HlyA can attack a wide spectrum of target cells; indeed, HlyA can even bind to synthetic planar lipid membranes, suggesting that binding is relatively nonspecific (203). Nonspecific adsorption to the cell surface, rather than high-affinity binding to a surface receptor, has also been proposed to precede membrane insertion of *B. pertussis* CyaA (133). Inactive (unacylated) precursor toxin does not inhibit cell binding by active, mature toxin for both HlyA and CyaA, compatible with there not being a saturable receptor (15, 133).

It is possible that receptor-ligand interactions do occur with some members of the toxin family, for instance LktA and AaltA, displaying a narrower range of target cell specificities. Competition between inactive LktA mutants and mature LktA has been demonstrated, suggesting that a receptor in the leukocyte membrane is involved in LktA target specificity (61), and LktA is unable to bind to bovine leukocytes pretreated with protease, suggesting the involvement of a proteinaceous component in toxin binding (42). AaltA has been suggested to use a β-integrin as a receptor, since it is able to bind both subunits of the lymphocyte function-associated antigen type 1. HlyA may use the same β_2-integrin for recognition of human leukocytes, but no direct binding has been shown (176). Integrins, a large family of αβ heterodimeric transmembrane receptors that are differentially expressed by a variety of cell types, have the potential variability to distinguish both cell type and species (except in erythrocytes) and are used as receptors during *Shigella* and *Yersinia* invasion of mammalian cells (84, 85, 130). Protein sequences potentially involved in putative receptor recognition would appear most likely to lie within the nonconserved domains of the toxin molecules (do the differences between *E. coli* HlyA and Ehx define regions of interaction with a human leukocyte receptor?). A model could be put forward in which toxin action against erythrocytes is an intrinsic property and does not use a receptor while action against leukocytes is defined by specific receptor interactions. The involvement of target cell components such as integrins would be consistent with the lack of receptor involvement in erythrocytes.

Subversion of Signal Transduction and Cytokine Production

Pathogenic bacteria frequently subvert host cell functions such as signal transduction pathways, cytoskeleton rearrangement, and vacuolar trafficking (reviewed in references 84 and 85). For example, enteropathogenic *E. coli* (EPEC) secretes proteins such as EspA and EspB that stimulate host phospholipase C, inducing inositol triphosphate production, the mobilization of intracellular Ca^{2+} stores, tyrosine phosphorylation of host proteins, and cytoskeleton rearrangement (251). Cell entry by *Yersinia*, *Listeria*, *Salmonella*, and *Shigella* also involves the activation of host signalling and a more extensive cytoskeleton rearrangement, which is controlled by small GTP-binding proteins belonging to the Ras family, namely, Rac, Rho, and CDC42. *Shigella*, *Listeria*, and *Yersinia* phosphorylate host proteins, including substrates for the nonreceptor tyrosine kinase Src. *Salmonella*, *Mycobacterium*, *Chlamydia*, and *Legionella* reside in intracellular membrane-bound vacuoles, and the stability of these vacuoles is believed to result at least in part from the bacteria interacting with vesicular trafficking that is normally mediated by a family of small GTP-binding proteins called Rabs. Proteins of the Ras, Src, and Rab families will be highlighted again in this review because of their possession of fatty acyl groups. There are also many examples of bacterial molecules inducing cytokine synthesis in eukaryotic cells, and although the most widely studied are lipopolysaccharide (LPS), capsular polysaccharides, and peptidoglycan, they also include porins, fimbrial proteins, heat shock proteins, and lipoproteins, as well as extracellular proteins such as proteases and toxins (reviewed in reference 120). Both gram-positive and gram-negative pathogens produce toxins that induce cytokine release; e.g., interleukin-1 (IL-1) and tumor necrosis factor (TNF) are induced in murine macrophages and human monocytes in response to listeriolysin O, pneumolysin, *C. difficile* toxin B, and *Streptococcus pyogenes* erythrogenic toxin A. Cholera toxin increases IL-6 synthesis and decreases TNF-α production by rat peritoneal mast cells, pertussis toxin enhances IL-4 production, and verotoxin induces the release of IL-1α, IL-6, and TNF-α in mouse macrophages.

Erythrocytes exposed to *E. coli* HlyA rapidly undergo cytoskeleton rearrangement, resulting in the formation of teardrop-shaped projections from the surface (147). HlyA also affects cytokine production. At very low, sublytic concentrations, HlyA is a potent trigger of G-protein-dependent generation of inositol triphosphate and diacylglycerol in granulocytes and endothelial cells, stimulating the respiratory burst and the secretion of vesicular constituents (30, 103). HlyA stimulates the release of IL-1β and TNF from human monocytes, the lipoxygenase products leukotriene B_4 and 5-hydroxyeicosatetraenoic acid and nitric oxide from endothelial cells, and IL-1β (but not TNF-α) from cultured monocytes, while it inhibits the release of IL-1β, IL-6, and TNF-α from human leukocytes (29, 102, 164, 295). When injected into mice, HlyA produces a cytokine response similar to those seen in vitro, elevating the levels of IL-1 and TNF in serum (200). This ability to affect cytokine release is shared by all the members of the HlyA toxin family; in vitro, LktA and AaltA reduce the lymphocyte response to various stimuli (195, 197), at low concentrations, LktA stimulates the production of IL-1 and TNF by bovine mononuclear phagocytes (287, 326) and potentiates the production of histamine and certain eicosanoids in response to chemokines (1). LktA and ApxII activate bovine and porcine neutrophils, respectively (63, 121, 306). These effects may occur without necrotic cell death, since HlyA and AaltA cause apoptosis of human lymphocytes and LktA causes apoptosis of bovine mononuclear cells and granulocytes (142, 197). As discussed below, some inflammatory cytokines possess a comparable acyl modification to that of HlyA.

E. coli HlyA and other RTX toxins alter the membrane permeability of host cells, causing lysis and death. Lysis of erythrocytes might provide bacteria with iron, and killing nucleated cells may prevent phagocytosis. The benefit to extracellular bacteria as a result of the induction of a host response is not so obvious. A consequence of the release of cytokines may be to induce inflammation, disrupt epithelial cell junctions, and favor translocation of bacteria through the intestinal barrier. The activation of host cell signal transduction pathways may also be advantageous to bacteria if the host responds by presenting a receptor used by them for binding. Such mechanisms are used by *B. pertussis*, where binding of filamentous hemagglutinin to a monocyte integrin causes the presentation of a second filamentous hemagglutinin-binding site (131), and by EPEC, where adherence to epithelial cells occurs after tyrosine phosphorylation by the host of an inserted bacterial protein, which then associates directly with intimin, an EPEC adhesin (160).

It is possible that some of the biological effects so far assigned to the HlyA toxin actually reflect cooperative responses to both toxin and LPS. In vivo and in vitro studies indicate that exposure to LPS, or mediators stimulated by LPS, increases the subsequent response to HlyA and LktA (261, 287). One

response of host cells attacked by HlyA is the rapid and massive shedding of LPS receptors (CD14), which may then bind to neighboring cells and make them sensitive to LPS. Such a mechanism might underlie the long-range detrimental effects of pore-forming toxins in host organisms (312). The induction of a host response may not be an alternative to cell death but an alternative pathway to death, proceeding via programmed cell death or apoptosis instead of lysis. Many bacterial toxins as well as members of the HlyA family can induce apoptosis (126). Host cell death by apoptosis would occur without leakage of cellular components, without inflammation, and without damage to surrounding cells (4).

Pore Formation in Eukaryotic Membranes

E. coli HlyA appears to have a two-stage interaction with eukaryotic membranes: a reversible adsorption sensitive to electrostatic forces and an irreversible insertion (10, 233). Transition to the inserted form is associated with a change of conformation (211) and is favored by membranes with a fluid state and low cholesterol content, which may explain earlier findings that membranes made from asolectin, a crude lipid mixture from soybean, are very sensitive to HlyA, in contrast to membranes composed of pure lipids such as phosphatidylcholine or phosphatidylserine (21, 231). Once inserted into a membrane, HlyA behaves as an integral protein; it cannot be extracted without the use of a detergent (27). HlyA causes target cell lysis by forming pores which display cation selectivity and voltage and pH dependence, as shown in experiments with whole cells, planar lipid membranes, and liposomes (203, 204). HlyA pores in erythrocytes are thought to be asymmetric, since proteolytic enzymes digest the toxin only when applied to one side of the membrane. Similarly, when monoclonal antibodies raised against HlyA were applied from the same side as the toxin, they could open or close the pore, but they could not do so when applied from the opposite side (204, 205). The physical properties of the transmembrane pore formed by *E. coli* hemolysin have been determined by using artificial lipid bilayers; it has a diameter of about 1.0 nm, a conductance of about 500 pS in 0.15 M KCl, and a mean lifetime of 2 s at low transmembrane voltages (21). HlyA pores in erythrocytes have been similarly sized, with a predicted physical cutoff of around 2 kDa (27). Patch-clamped human macrophages have been used as targets for HlyA, and this has confirmed that the leukotoxic action of HlyA is also due to the formation of pores with very different properties from the endogenous pores already present in the cell membrane (204). Similar physical properties have been assigned to the pores formed by other members of the HlyA toxin family (22, 55, 194, 203, 258), suggesting that pore formation is a closely conserved step in the action of these toxins.

For many bacterial toxins unrelated to *E. coli* HlyA, pore formation is synonymous with oligomerization, with their structures varying from trimer to 100-mer. *V. cholerae* El Tor cytolysin may form 3- to 5-mer oligomeric structures (206). *E. coli* heat-labile enterotoxin and *P. aeruginosa* cytotoxin form pentameric pores (228, 307), while aerolysin and *S. aureus* alpha-toxin oligomerize to produce heptameric pores (236, 278). The two gram-positive toxins streptolysin O and pneumolysin polymerize to form pores with a large and variable oligomeric superstructure of between 25 and 100 monomers (214, 235).

Oligomeric forms of staphylococcal alpha-toxin and streptolysin O are unusually stable and can be extracted intact from membranes with detergent (26, 90). In contrast, HlyA recovered from deoxycholate-solubilized erythrocyte membranes is recovered in a monomeric form, indicating either that oligomerization is not required for pore formation or that oligomers are dissociated in the detergent (27). The lower stability, larger size, and lower solubility of HlyA-related toxins have made elucidation of the accurate subunit stoichiometry of the membrane-inserted pore even more complex and left it currently unresolved. As with streptolysin O and staphylococcal alpha-toxin, membrane insertion of HlyA and CyaA is believed to occur through a monomolecular mechanism (24, 286), with oligomerization, if any, occurring by the subsequent addition of monomers within the membrane. It has been estimated that only one to three HlyA molecules form the pore (21, 203), while CyaA pores in artificial lipid bilayers indicate a functional unit of a trimer or larger (297). The increases in membrane conductance on exposure to HlyA and Apx toxins have been explained by an association between nonconducting monomers and conducting oligomers (21, 194). The complementation of inactive deleted variants of HlyA to produce hemolytic activity and CyaA to produce cytotoxic activity has suggested that two or more toxin molecules aggregate before pore formation and substantiate the view that oligomerization is involved (133, 189). However, in the absence of a defined pore structure, it is worth bearing in mind that membrane disruption may occur through mechanisms other than formation of discrete pores. Examples are provided by the antibacterial peptide cecropin, where a "carpet" of peptide monomers disrupts phospholipid packing (93), and complement-mediated lysis by the formation of "leaky patches" in the lipid bilayer (327). Indeed, the exposure of membranes to toxin at high concentration or for long times may lead to similar lesions through a detergent-like mechanism (210, 231).

A highly conserved region (amino acids [aa] 238 to 410) of HlyA (20, 61) is essential for lysis and is believed to be involved in pore formation (Fig. 2). It spans the only pronounced hydrophobic sequences in the otherwise hydrophilic HlyA protein, and secondary-structure predictions suggest that it comprises four membrane-spanning α-helices, each of 21 aa. The sequences preceding and between these four putative HlyA α-helices have strong amphipathic α-helical properties and possess membrane-binding characteristics. It has been suggested that these repeated hydrophobic and amphipathic helices may insert into the cytoplasmic membrane and form a pore, allowing the influx of extracellular calcium and the escape of potassium (188, 203). Mutations altering the hydrophobicity of this region reduce or abolish the pore-forming activity of the protein on erythrocytes and artificial membranes (186–188).

The conserved glycine-rich repeat domain associated with HlyA Ca^{2+} binding is required for the hemolysis of erythocytes but not for pore formation in asolectin lipid bilayers, since its deletion leaves the pore-forming ability of HlyA unaffected (187) (Fig. 2). In addition, HlyA shows full channel-forming activity in artificial lipid bilayers even in the presence of 5 mM EDTA (74). This suggests that while Ca^{2+} binding is critical at some stage of the lytic process such as promoting the irreversible insertion of the toxin into the membrane (11), it does not directly contribute to the pore-forming structure (37, 187). It also suggests that the asolectin lipid bilayer system is not a true reflection of binding and pore formation in real cell membranes, a conclusion supported by the finding that pore formation in asolectin bilayers is also independent of acylation (190). The conformation of the repeat region, which remains on the same side as that from which the toxin approached the membrane, may affect the state of the pore since the binding of a monoclonal antibody to this region led to pore closure (205).

Mutations in the LPS biosynthetic genes affect the expres-

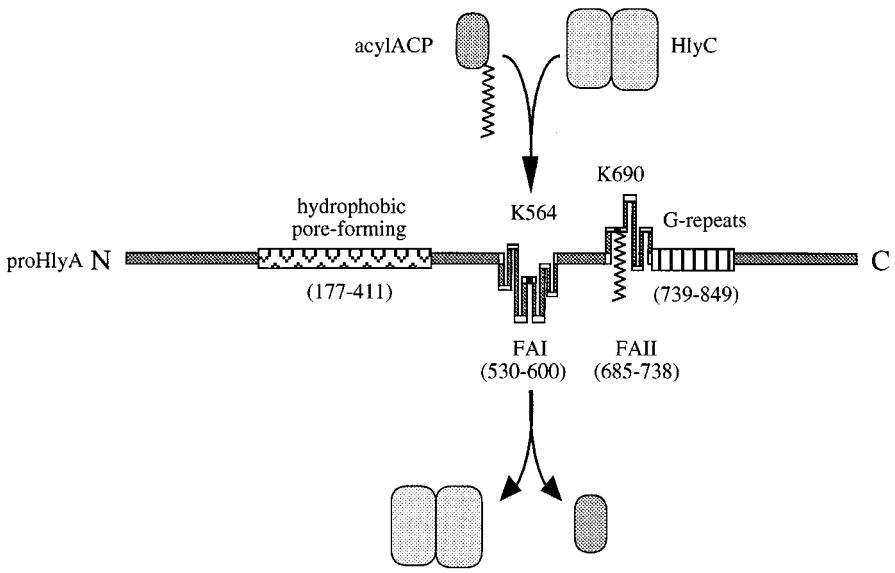

FIG. 2. Maturation of pro-HlyA to the active toxin by HlyC and acyl-ACP. The positively charged HlyC homodimer associates with the negatively charged ACP. Following binding to the HlyC recognition domains, FAI and FAII, the acyl chain is transferred to the corresponding acyl modification sites, K564 (KI) and K690 (KII). The acylated lysines KI and KII are shown relative to the hydrophobic pore-forming domain and the glycine-rich Ca^{2+}-binding repeats of pro-HlyA.

sion and/or activity of *E. coli* HlyA (17, 286, 314). A transposon insertion in *rfaP* (required for attachment of phosphate-containing substituents to the LPS inner core) reduces the extracellular activity but not the export of HlyA as a consequence of the formation of large extracellular toxin aggregates; biological activity is restored with chaotropic agents (286). Why this occurs is not clear, but LPS possesses calcium-binding sites that may act as outer membrane reservoirs of calcium, and alterations in LPS structure may reduce the availability of calcium, resulting in the secretion of an inactive, unstable HlyA prone to aggregation. Alternatively, direct contact between wild-type LPS and HlyA may prevent toxin aggregation.

None of the dramatic effects of HlyA on eukaryotic host cells outlined above occur in vivo if the toxin is produced in the absence of the cotranslated protein, HlyC. The elucidation of the reason for this strict requirement has revealed a mechanism of bacterial toxin maturation unknown outside the HlyA family.

ESSENTIAL MATURATION OF HlyA: A UNIQUE FATTY ACYLATION

Bacterial Mechanism for Modifying Lysine Residues

HlyA toxin is synthesized as an inactive 1,024-residue protoxin, pro-HlyA, which is activated intracellularly to the mature toxin by the action of the cosynthesized HlyC. It was shown by using an in vitro system that this maturation is a fatty acylation and that HlyC is a homodimeric putative acyltransferase, using acyl-acyl carrier protein (acyl-ACP) as the fatty acid donor (112, 128, 129, 132, 282, 283) (Fig. 2). In in vitro reaction mixtures containing only purified acyl-ACP, HlyC and pro-HlyA, the acquisition of hemolytic activity is directly related to the binding of fatty acid by pro-HlyA. Mass spectrometry and Edman degradation of proteolytic products from mature HlyA toxin activated in vitro by HlyC and [^{3}H]acyl-ACP revealed two fatty-acylated internal lysine residues, K564 (KI) and K690 (KII), and resistance of the acylation to hydroxylamine suggested that the fatty acid is amide linked. Substitution of the two lysines confirmed that they are the only sites of acylation and showed that although each is acylated in the absence of the other, both sites are required for in vivo toxin hemolytic activity (282). The K564 and K690 residues were subsequently confirmed to be modified in in vivo-synthesized and secreted HlyA by peptide mapping and two-dimensional electrophoresis (190). The internal pro-HlyA modification explains the earlier isolation of a monoclonal antibody that recognized only the active form of HlyA, specifically epitope aa627 to 726 (238).

The other HlyA-related toxins secreted by pathogenic gram-negative bacteria all require HlyC-type protoxin activation, and indeed many (PvxA, MmxA, ApxIA, and LktA) have been activated by *E. coli* HlyC (87, 106, 167). In addition, CyaC, ApxIIC, and AaltC are able to activate pro-LktA (175, 202, 322). CyaA, the adenylate cyclase hemolysin of *B. pertussis*, requires posttranslational activation both to deliver its catalytic domain into the mammalian target cell (cell-invasive activity) and to form transmembrane channels (hemolytic activity). It has been demonstrated by mass spectrometry that CyaA toxin secreted by *B. pertussis* is modified by amide-linked palmitoylation on the ε-amino group of K983 (analogous to HlyA KII, K690) (108). Only modification sites of HlyA and CyaA have been demonstrated to be acylated, although the loss of activity caused by deletion of the region between aa 358 to 548 of *P. haemolytica* LktA is compatible with the KI residue, K554, as a potential site of modification (61), and activation of a CyaA-LktA hybrid toxin supports the notion that the region aa 379 to 616 of LktA is important for LktC recognition (322). Substitution of LktA K554 (to T and C) reduced the lytic activity of LktA against bovine lymphocytes by only ca. 40%, but the retention of lytic activity does not rule out K554 as an LktA acylation site, since HlyA mutants with either KI or KII deleted also retained about 50% activity against BL-3 cells despite being inactive to erythrocytes (239, 282). Bovine lymphocytes may be less sensitive to the state of acylation of these toxins than are either erythrocytes or human lymphocytes, but it remains a possibility that LktA contains an acylation site different from the KI and KII sites identified for HlyA and CyaA. No data for the other toxins have been presented.

HlyC, a Novel Acyltransferase

The ability to transfer an acyl group to an internal lysine residue of a target protein distinguishes HlyC from all other bacterial acyltransferases, and *hlyA*$^+$ *hlyC* strains are nonhemolytic, indicating that the various constitutive acyltransferases of *E. coli* are unable to substitute for HlyC to even a small degree. HlyC has no significant sequence homology to known acyltransferases such as the lipid A acyltransferases (3, 54, 56, 158), the *Rhizobium* Nod factor acyltransferases NodL and NodA (35, 69), or the well characterized acyltransferases such as glycerol-3-phosphate acyltransferases (182) or eukaryotic *N*-myristoyltransferases (140, 332). HlyC may therefore be an acyltransferase that is structurally and functionally distinct from all other acyltransferases.

Little is known about the biochemical properties of HlyC, the enzyme responsible for protoxin acylation. Bacterial and eukaryote acyltransferases generally accept either acyl coenzyme A (acyl-CoA) or acyl-ACP as an acyl donor, but ACP is a strict requirement for HlyC-directed pro-HlyA acylation (acyl-CoA cannot be used). Myristoyl-ACP gave the highest hemolytic activity of HlyA acylated in vitro with a range of fatty acids (C_{12} to $C_{18:1}$) (132), which is consistent with an apparent selection of myristoylation at both the KI and KII sites in vitro. The affinity of KI for myristoyl-ACP (a saturated acyl group of 14 carbons) in vitro is approximately twice that for palmitoyl-ACP (a saturated acyl group of 16 carbons), while the affinity of KII for myristoyl-ACP is approximately five times that for palmitoyl-ACP (285). In vivo, both K564 and K690 appear fully acylated, suggesting that unlike CyaA, HlyA is predominantly myristoylated (190). Not only is HlyC able to discriminate between acyl-ACPs carrying fatty acids of different lengths but also it is presumably able to do this at low substrate concentrations, since *E. coli* does not have significant pools of acyl-ACPs with fatty acids longer than 3 carbons (249). In vitro measurements of K_m for both ACP and protoxin substrates suggest that HlyC does indeed recognize its substrates at extremely low concentrations (~100 and ~10 nM, respectively). HlyC is able to bind tightly but, it seems, noncovalently both the acyl chain from acyl-ACP and phosphopantetheine, but the locations of the active site of HlyC and regions that bind its acyl-ACP and pro-HlyA substrates are not known (284). The specificity for acyl-ACP as the acyl donor is shared in *E. coli* by the acyltransferases involved in lipid A biosynthesis, LpxA, LpxD, HtrB, and MsbB, but the use of a protein as a substrate is unique to HlyC (43, 158, 246). The cytosolic enzyme, LpxA, catalyzes the first step of lipid A biosynthesis, transferring (*R*)-3-hydroxymyristate from ACP to UDP-*N*-acetylglucosamine (3). LpxD is also specific for (*R*)-3-hydroxymyristoyl-ACP (158). The so-called "late" acyltransferases, HtrB and MsbB, can both use lauroyl-ACP and myristoyl-ACP as substrates for generating acyloxyacyl residues in lipid A, although HtrB shows a fivefold preference for lauroyl-ACP (54).

HlyC Recognition Domains on the Protoxin

By using deleted protoxin variants and protoxin peptides as substrates in an in vitro maturation reaction dependent on only HlyC and acyl-ACP, two independent HlyC recognition domains (FAI and FAII) have been identified on the HlyA protoxin, each of which spans one of the target lysine residues, KI or KII, respectively (283) (Fig. 2). Each domain requires 15 to 30 aa for basal (ca. 10%) recognition and 50 to 80 aa for full wild-type acylation. The peptide recognition sequences for HlyC appear to be larger than those of other acyltransferases, since acylation of mutagenized target substrate proteins, chimeric proteins, and substrate peptides has indicated that peptide sequences of 4 to 15 aa are recognized by *N*-myristoyltransferases and prenyltransferases (198, 240, 302, 303, 331). However, these modification sites are generally at the N and C termini of the substrate proteins, where they may be relatively open and accessible. In contrast, the internal acylation sites of HlyA may require HlyC to recognize a larger topology rather than a linear sequence. While the HlyA FAI domain is symmetrically centered on the modified lysine residue, the FAII domain appears to be asymmetrical, not requiring amino acids N-terminal to the modified residue (283). The two domains are nevertheless functionally indistinguishable and compete with each other for HlyC both in *cis* and in *trans*. No other HlyA sequences are required for toxin maturation, including the immediately C-terminal Ca^{2+}-binding repeats (aa 722 to 849). Indeed, in vitro, Ca^{2+} ions prevent acylation at both the KI and KII sites (283). The extreme sensitivity of the pro-HlyA activation reaction to free Ca^{2+} supports the view that intracellular Ca^{2+} levels in *E. coli* are too low to affect toxin activity (92) and that Ca^{2+} binding does not occur until the toxin is outside the cell.

The HlyC recognition sites of *E. coli* HlyA have been compared with each other and with the corresponding sequences of other members of the toxin family. Although FAI and FAII of HlyA have the same function, there is little primary sequence identity (only 21%), and this lack of homology is also evident within each site among the toxins; only 20% of the FAI residues and 17% of the FAII residues are identical in the five toxins shown (Fig. 3). This divergence may reflect differences in the HlyC-type proteins, the pattern of acylation at the two sites (where present, some toxins of the *Pasteurellaceae* family lack the KII target lysine), and the preferred target cell range of each toxin. Consensus sequences constructed for each of the two acylation sites have little in common apart from the central "GK" motif, suggesting that similarity between FAI and FAII may be of a higher structural order. Secondary-structure predictions of FAI and FAII indicate that both regions are rich in β-turns, quite regularly spaced approximately every 10 aa, especially in FAI. Other predicted features are not shared by FAI and FAII, except perhaps for a helical region immediately N-terminal to both KI and KII. However, helical-wheel projections do not show amphipathic distributions of charged or hydrophobic residues as related to membrane-associated structures. The lack of identity between FAI and FAII domains in pro-HlyA and corresponding sites on related protoxins currently deters an explanation for the basis of HlyA recognition by HlyC.

A lack of cross-complementation between protoxin activator C proteins supports the view that C-proteins may be unable to modify a KI or KII site even when it is present. *E. coli* HlyC is able to activate *P. haemolytica* LktA, whereas LktC is unable to activate HlyA and CyaA (87, 322). One explanation would be that LktC can acylate only KI even when KII is present. However, this explanation could not extend to *A. pleuropneumoniae* ApxIIC, which can activate *P. haemolytica* LktA, whereas LktC is able to activate ApxIIA only marginally, despite both toxins apparently possessing only KI (202) (Table 3). It seems possible that there are additional factors influencing the recognition between protoxin and HlyC-like activator proteins. One such additional factor appears to be the host cell in which the toxin operon is expressed. In contrast to the native CyaA protein from *B. pertussis*, which is palmitoylated exclusively at K983 (KII), recombinant pro-CyaA protoxin modified by CyaC in *E. coli* is acylated at K983 and contains an additional acylation site at K860 (analogous to KI) (109). It is perhaps this anomalous acylation in *E. coli* that allows CyaC to activate LktA (322). The specificity of CyaC peptide recognition may be

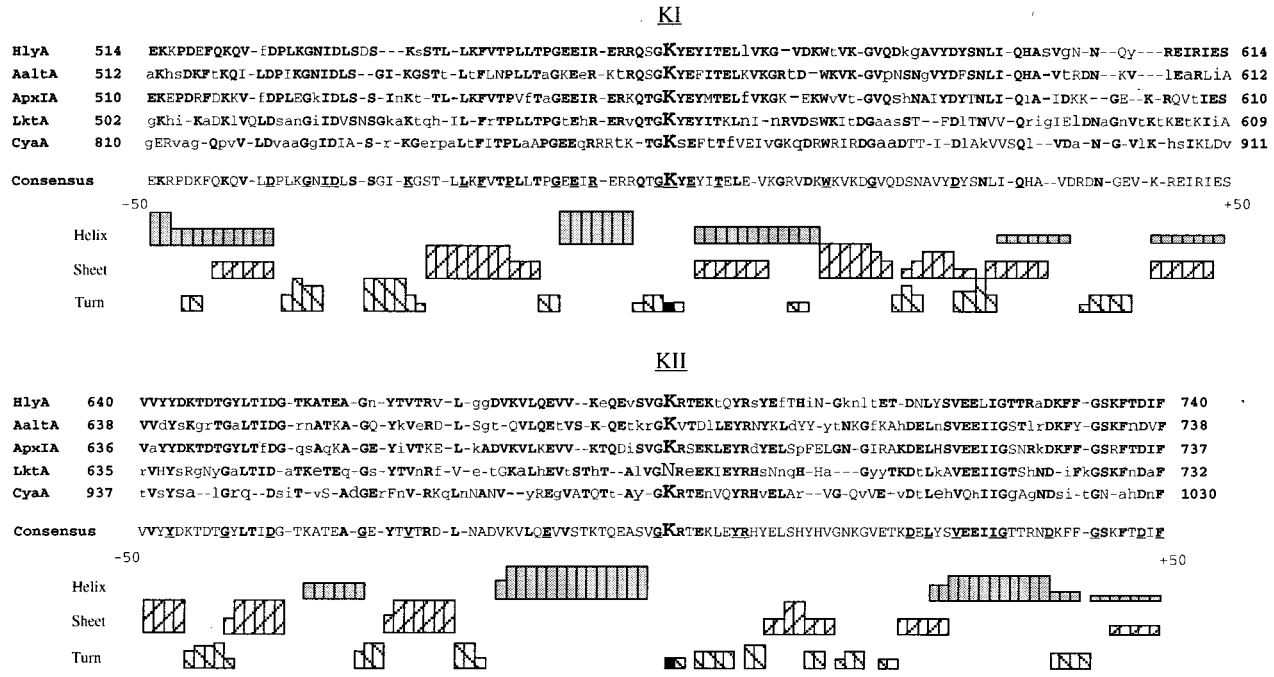

FIG. 3. Alignment of the *E. coli* HlyA FAI and FAII HlyC recognition domains with corresponding sequences of the related toxins *A. actinomycetemcomitans* AaltA, *A. pleuropneumoniae* ApxIA, *P. haemolytica* LktA, and *B. pertussis* CyaA. Identical residues are in boldface type; amino acids belonging to the same class are in capitals and non-homologous amino acids are in lowercase. Hyphens represent breaks introduced to maximize identity. Consensus sequences for FAI and FAII indicate residues shared by four toxins (boldface) and by all five toxins (underlined). A cumulative histogram representation of secondary structure predictions by the Chou and Fasman and the Robson and Garnier methods are shown under the primary sequences.

affected by the host cell ACP, but this presumes that it is a C-ACP complex which recognizes the toxin. Such a mechanism is supported for HlyC by in vitro kinetic data that also indicate the involvement of a ternary HlyA-HlyC-ACP complex during the transfer of fatty acid from acyl-ACP to pro-HlyA (284). An ordered Bi-Bi mechanism would be analogous to that of *N*-myristoyltransferases, for which it is the binding of acyl-CoA which determines subsequent peptide binding (252, 332). This might explain the earlier failures of LktC to activate the proforms of HlyA, ApxIIA, and CyaA (87, 202, 322) in experiments which were performed in or with extracts from an *E. coli* background. Alternatively, *E. coli* may possess an escort protein, similar to that observed with prenylated proteins, which enhances the acylation at KI but which is absent in *B. pertussis* (269). However, one might expect such an escort protein to have been identified by mutagenesis studies carried out in many laboratories that have successfully identified other accessory proteins, such as TolC and RfaH, required for toxin activity. Although the data available at present are incomplete and address only HlyA and CyaA, it appears that there is selection for the fatty acid attached to the toxin and that *E. coli* and *B. pertussis* have evolved hemolysin acyltransferases with different affinities for fatty acid. The *E. coli* HlyC protein may preferentially acylate HlyA KII with myristic acid, whereas the *B. pertussis* CyaC protein preferentially acylates CyaA KII with palmitic acid. Similar variations in substrate specificity between related proteins of different species appear between *E. coli*, *Neisseria meningitidis*, and *P. aeruginosa* LpxA, which are specific for (*R*)-3-hydroxymyristoyl-ACP, 3-hydroxylauroyl-ACP, and 3-hydroxydecanoyl-ACP, respectively (75, 227), and *E. coli* and *Haemophilus influenzae* HtrB, which are specific for lauroyl-ACP and myristoyl-ACP, respectively (54, 224). Not only do differential fatty acid affinities require an explanation, but so do cross-species complementation tests with C-proteins and host-specific acylation patterns. Presumably, these explanations will lie in the differences between the structures of the family toxins, activator C-protein acyltransferases, and ACPs.

An insight into the modification of HlyA might be expected from an examination of other examples of protein lipidation. We present an extensive review of these and show that while the different types of modification vary considerably in terms of the location and structure of the lipid moiety, it is possible to define universal themes in terms of their function. Despite the absence of data for acylation events exactly equivalent to that of the prokaryotic toxins, it is still possible to draw conclusions from the more extensively studied examples that are relevant to the roles that toxin acylation may play in determining biological function. Lipidated proteins found in and on eukaryotic membranes are included in this comparison since the eukaryotic cell membrane is the site of action of HlyA and its related toxins. It is also becoming apparent that these acylated proteins are the sites of action of many bacterial effector proteins either directly by modification (e.g., ADP-ribosylation, glucosylation, and deamidation of small G-proteins and heterotrimeric G-protein subunits) or indirectly by interfering with their enzymatic pathways (e.g., protein phosphatases and kinases) (84, 85).

PROTEIN LIPIDATION IN PROKARYOTES AND EUKARYOTES

Lipidation is involved in the maturation of many proteins in both prokaryotic and eukaryotic cells, including viral oncogene products, but it is achieved by various mechanisms which differ according to the fatty acid transferred, the amino acid modified, and the fatty acyl donor. Myristic and palmitic acids are

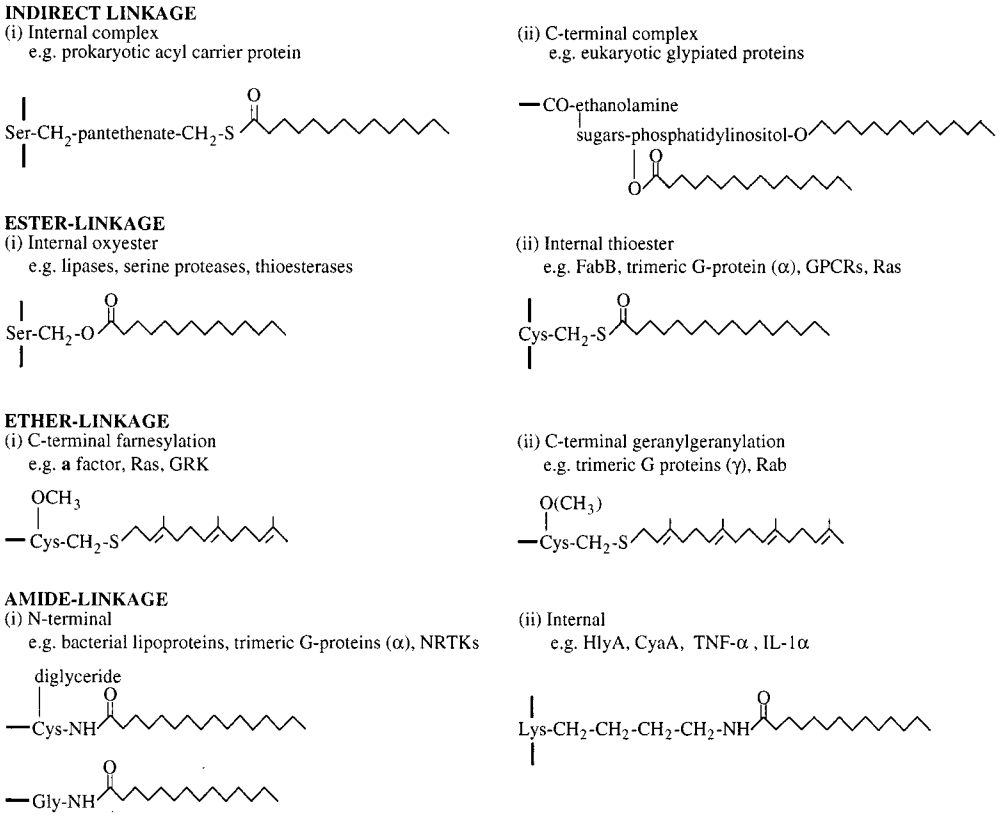

FIG. 4. Major classes of lipidated proteins in prokaryotes and eukaryotes. The structure of the lipid and their attachment to the protein peptide backbone are shown, except for the complex indirect linkages of pantetheinylated and glypiated proteins. For heterotrimeric G-proteins, the particular modified subunit is indicated in parentheses. The placement of the methyl group of geranylated proteins in parentheses indicates that carboxymethylation is not universal.

the most common fatty acids cross-linked to proteins. Proteins sorted to the bacterial outer membrane or eukaryotic plasma membrane undergo processing in which an acyl group is attached to the N-terminal amino acid; enzymes with acyltransferase, lipase, or esterase activity use catalytic mechanisms involving ester-linked acyl groups attached to serine and cysteine residues; and eukaryotic proteins use ester-linked palmitoylation and ether-linked prenylation of cysteine residues for membrane sorting and protein-protein interaction. As can be seen below, the acylation of pro-HlyA does not equate to any of these but instead appears to be limited to its related toxins and perhaps a handful of eukaryotic proteins (Fig. 4).

Lipid Groups Indirectly Linked to Their Peptide Backbone

Bacterial phosphopantetheinylated proteins. The inclusion of a phosphopantetheine cofactor increases the number of thiol groups on a protein and introduces a tether to increase the flexibility of acyl chains between various active sites. As one of the most abundant proteins in *E. coli*, constituting 0.25% of the total soluble protein, ACP is an important example of a protein carrying an indirectly linked acyl group. In addition, the central involvement of ACP in the acylation of bacterial toxins warrants an extended account of this small but multifaceted protein. ACP is required throughout fatty acid biosynthesis, carrying fatty acids as thioester intermediates attached to the terminal sulfhydryl of a 4′-phosphopantetheine group (4′-PP), which is in turn attached to a serine residue (Ser36) via a phosphodiester linkage (192).

E. coli ACP is a 9-kDa acidic protein (pI 4.1). The *acpP* structural gene has been cloned and overexpressed (143), and the ACP solution structure has been defined (127). Sequences between aa 31 and 71 are thought to be involved in fatty acid binding, with the acyl chain held in a pocket (144, 201). The 4′-PP sulfhydryl is the only thiol group in *E. coli* ACP, and without it the apoprotein is inactive. Holo-ACP synthase (encoded by *acpS*, formerly *dpj*) transfers the 4′-PP moiety from CoA to apo-ACP to produce holo-ACP (178). In normally growing cells, virtually all of the ACP is maintained in the active, holo-form (134, 156). During logarithmic growth, there is a significant pool of unacylated holo-ACP together with acetyl-ACP and malonyl-ACP, but there is no acyl-ACPs of four carbons or longer (249).

As well as being a component of the fatty acid synthase and acting as an acyl donor for the HlyA family of protein toxins (132), *E. coli* ACP functions in the transfer of long-chain fatty acids to phospholipids (58, 249) and to the lipid A component of LPS (3). In *E. coli* (and mitochondria), ACP is used by lipoate transferases as a lipoate donor (146). In addition, ACP is tightly associated with MukB, a protein required for chromosome partitioning (226), and it may also be involved in the initiation of the transposition of Tn*3* (191). In a role known not to require the 4′-PP group, ACP is an essential component of a UDP-glucose-requiring transglucosylase system that catalyzes the synthesis of the β-1,2 backbone of membrane-derived oligosaccharides (299). Homologs of *E. coli* ACP occur as integral domains of large multifunctional enzymes such as in the eukaryotic fatty acid synthases and in multiprotein complexes as discrete proteins. Apart from playing a role in fatty acid synthesis, ACP or ACP-like proteins are needed in the

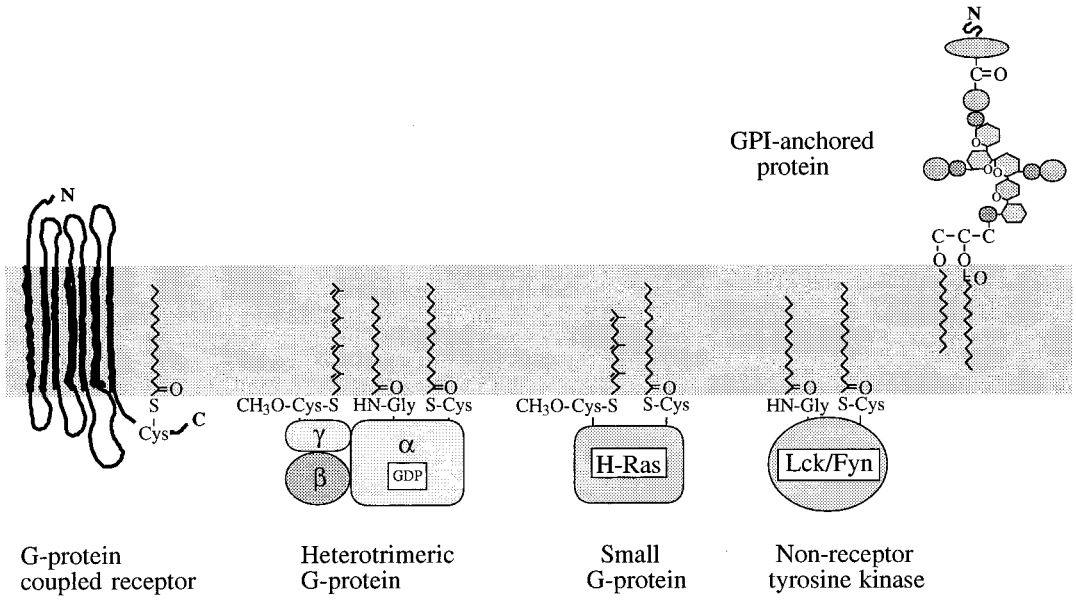

FIG. 5. Membrane localisation of lipidated proteins on the plasma membrane of mammalian cells targeted by HlyA. Typical proteins from the major types involved in cell signalling are represented. The lipid groups are shown inserted directly into the bilayer, although other protein interactions may be involved. Many G-protein coupled receptors possess internal cysteine residues that may act as palmitoylation sites as shown, and some have been shown to be modified. The heterotrimeric G-protein α subunit, Ras and NRTK proteins shown on the inner face of the plasma membrane possess two lipids, although other isotypes may have only one. The G-protein α-subunit and Src-related NRTK contain both a myristoyl group linked to the N terminus and a thioester-linked palmitoyl group. The G-protein γ subunit contains a C-terminal thioether-linked geranylgeranyl isoprenoid. The Ras-related small G-protein contains a C-terminal thioether-linked farnesyl isoprenoid and a thioester-linked palmitoyl group. The structure of a typical GPI-anchored protein with its constituent inositol, glucosamine, mannose, and ethanolamine groups is shown. An additional palmitoyl chain may be attached to the inositol ring.

synthesis of polyketide (e.g., *Streptomyces glaucescens* 9-kDa TcmM [268]), nonribosomally synthesized peptide (e.g., *S. clavuligerus* 300-kDa ACV synthetase [12]), and depsipeptide (e.g., *Fusarium scirpi* 347-kDa enniatin synthetase [241]), cell host signalling (e.g., *Rhizobium* 10-kDa NodF [95]), and cell wall synthesis in gram-positive bacteria (e.g., *Lactobacillus casei* 6-kDa Dcp [118]). In bioluminescent bacteria such as *Vibrio harveyi*, ACP is also involved in the synthesis of the myristaldehyde substrate used by luciferase (47).

Only one ACP has been found in *E. coli*, and this appears sufficient for all ACP-dependent roles. In contrast, *Rhizobium* species make not only a constitutive ACP but also the inducible NodF, which participates in the transacylation of oligosaccharides for nodulation (243, 267), and AcpXL, which donates 27-hydroxyoctacosanoic acid to a lipid A precursor (44). Three ACPs have also been identified in *S. coelicolor*; one is presumed to be the fatty acid synthase ACP, while the other two are involved in the synthesis of aromatic polyketides (248). The anonymous gene *iacP* of *Salmonella typhimurium* SPI-1 (*Salmonella* pathogenicity island 1) encodes an ACP that may be specific for bacterial invasion, with an as yet unidentified ACP being responsible for the synthesis of essential phospholipids, as in *E. coli* (151). The importance of these specialized bacterial ACPs may be to allow the synthesis of unusual metabolites without interfering with the synthesis of essential cellular lipids. In a typical bacterial cell, as many as a dozen different enzymes, with overlapping acyl chain specificities, compete for the pool of acyl-ACP. Consequently, variations in the structure of ACP could have a large influence on the metabolic fate of acyl groups, including their transfer to acylated bacterial toxins.

Apart from ACP, *E. coli* possesses at least two other proteins that contain a 4′-PP group and so potentially have indirectly linked acyl groups. EntB is a 33-kDa protein that serves as an aryl carrier protein, and EntF is a 142-kDa enzyme that is responsible for serine activation during the biosynthesis of enterobactin (94, 253). Gramicidin S synthetase, a *Bacillus* enzyme with homology to EntF, carries 4′-PP on a serine residue within a conserved LGGDSI motif (310). Three phosphopantetheinyl transferases have been identified in *E. coli*. ACP synthase is specific for ACP, EntD modifies EntB and EntF, and the substrate for the third is unknown (94, 177).

Eukaryotic glypiated proteins. Fatty acyl groups are linked indirectly to many eukaryotic proteins by the GPI moiety (Fig. 5). This contains an entire phospholipid, in which the lipid moiety is variable, possessing C_{14} to C_{24} acyl groups, and is associated with sugars and ethanolamine (77). In many instances, the inositol ring contains an additional lipid modification in the form of an ester-linked palmitic acid. The complete GPI anchor is transferred to proteins by GPI-transamidases at a specific C-terminal recognition sequence which is cleaved in the process. The GPI signal sequence is extremely degenerate but commonly features a run of 12 to 20 hydrophobic residues. GPI-anchored proteins are abundant on the cell surfaces of lower eukaryotes such as protozoa and yeasts and have a wide variety of functions such as nutrient uptake and membrane-signalling events; they include hydrolytic enzymes, receptors, cell adhesion molecules, complement inhibitors, and antigens of unknown function (77, 79, 98).

Lipid Groups Directly Linked to the Protein Peptide Backbone

Ester-linked acylation. The attachment of lipid groups to proteins through ester bonds provides a labile connection suitable for a transient role such as in catalytic mechanisms. Some enzymes in the biosynthetic pathways of fatty acids, phospholipids, and LPS bind acyl groups tightly but do not necessarily

form covalent acyl-enzyme intermediates, e.g., *E. coli* acyl-ACP synthase (136) and β-hydroxy-decanoyl-ACP dehydrase (180). However, other enzymes, particularly acyltransferases but also lipases and esterases, do use mechanisms involving oxy- and thioester acyl-enzyme intermediates (45, 66, 80, 138, 179, 181, 192, 193). In addition to these enzymes, a class of acylated proteins found in eukaryotes but apparently absent in prokaryotes carry ester-linked acyl groups not for a catalytic purpose but for structural reasons. The nature of this modification is outlined below, and its influence on protein structure and function is examined for lessons that may be applied to the acylation of HlyA.

The covalent modification of eukaryotic proteins with palmitate occurs posttranslationally at the thiol group of cysteine residues. A number of proteins involved in intracellular signalling are palmitoylated (208, 209, 216, 247). A wide variety of cell responses to signals such as growth factors, neurotransmitters and hormones is mediated by GTP-binding (G)-protein-coupled receptors (GPCRs), which are integral heptahelical proteins located on the plasma membrane. On ligand binding, these activate heterotrimeric G-proteins on the inner face of the membrane through direct interaction. Heterotrimeric G-proteins comprise three subunits, α, β, and γ, the last two forming a very tight complex (317). Many GPCRs, including the pigment rhodopsin (39, 321), some heterotrimeric G-protein α-subunits (216), receptor tyrosine kinases (RTKs) (290), small G-proteins (222), and non-receptor tyrosine kinases (NRTKs) of the Src family (247, 272) are palmitoylated (Fig. 5).

The labile nature of the thioester bond opens the possibility of acylation being reversible, and there is evidence for this in enzymes that catalyze both attachment and removal of lipid, which could regulate the palmitoylation state of a target protein (216). So far, these enzymes are largely uncharacterized. However, an assay for the palmitoyl acyltransferase that palmitoylates members of the Src family has been developed (23), enzymes that transfer palmitoyl groups to H-Ras and spectrin have been purified (67, 184), and a thioesterase that removes palmitate from H-Ras and Gα subunits has been cloned (50).

In contrast to the labile connection of ester-linked acyl groups, the ether linkage of isoprenoid groups and the amide linkage of acyl groups provide proteins with a stably attached lipid. Such irreversible modifications are potentially more relevant to the amide-linked maturation of toxins, although, again, the responsible transferases are distinct from HlyC.

Ether-linked prenylation. Several eukaryotic intracellular proteins are post-translationally modified by farnesyl (C_{15}) or, more commonly, geranylgeranyl (C_{20}) unsaturated isoprenoid lipids, attached through thioether bonds to cysteine residues at or near their C terminus (Fig. 5). This constitutive process results in a stably modified protein. Three protein prenyltransferases are responsible for the modification of separate substrate groups. Farnesyltransferase and geranylgeranyltransferase I recognize proteins with a -CaaX C-terminal motif. Following prenylation, the three C-terminal residues are removed from most CaaX-type proteins and the prenylcysteine residues is methylated at its exposed carboxyl group. Geranylgeranyltransferase II recognizes C-terminal double cysteine motifs such as -CC or -CxC (198, 331).

Isoprenylated proteins include fungal mating factors, nuclear lamins, several vesicular transport proteins, the oncogene product Ras and Ras-related GTP-binding proteins, the subunits of trimeric G-proteins, and protein kinases (48, 331). For a variety of fungi, mating is initiated by peptide pheromones, several of which have been suggested to be lipopeptides. In *Saccharomyces cerevisiae*, the **a**-factor is isoprenylated with a farnesyl group. Members of the Ras family of monomeric GTP-binding proteins are modified by isoprenoid lipids (198), and Ras itself is farnesylated. Geranylgeranyl and geranylgeranyl/farnesyl modifications are reported for mammalian Rab proteins and yeast Ypt proteins. The digeranylgeranylation of Rab proteins is a complex process, requiring a Rab geranylgeranyltransferase and an escort protein (REP). Each acylation is an independent reaction, but mono-GG-Rab remains bound to REP, ensuring the efficient double modification of Rab (269). Heterotrimeric G-protein γ subunits are prenylated (217). The geranylgeranyl moiety is found on all γ subunits except the retinal-specific form, which is farnesylated. G-protein-coupled receptor kinases may be modified with a farnesyl group (e.g., retinal G-protein-coupled receptor kinase) (234).

Amide-linked acylation. (i) N-terminally acylated bacterial lipoproteins. Lipoproteins exported to the periplasm or outer membrane of gram-negative bacteria are modified with diacylglycerol, via thioether linkage to cysteine, and with an amide-linked fatty acid (65% palmitate) on the N terminus (116). The precursor protein undergoes three reactions directed by enzymes in the inner membrane (i.e., phosphatidylglycerol diacylglyceryl transferase, signal peptidase II, and apolipoprotein *N*-acyl transferase), resulting in the generation of *N*-acyl diacylglycerylcysteine as the N-terminal amino acid (254). The first known example of bacterial lipoprotein, *E. coli* murein (also called Braun's lipoprotein), occurs as a free form and a bound form covalently attached to the peptidoglycan layer by a peptide linkage via the ε-NH_2 group of the C-terminal lysine. The fatty acids transferred to the prolipoprotein are taken from the phospholipid pool, with the major source being phosphatidylethanolamine (104, 135). Prolipoprotein acylation produces lysophospholipids that are reacylated by the inner membrane 2-acyl-glycerophosphoethanolamine acyltransferase. This acyltransferase can use acyl-ACPs from fatty acid biosynthesis or can convert fatty acid to acyl-ACP in the presence of ATP-Mg^{2+} (58).

Bacterial proteins with a broad range of functions have been found to be lipidated. More than 130 direct homologs of Braun's lipoprotein have been identified in gram-negative bacteria, including extracellular enzymes (e.g., β-lactamases, chitobiase, and β-1,4-endoglycanase); TraT, a surface-exposed lipoprotein that blocks conjugative transfer to cells carrying related plasmids (113); PulA, the pullulanase of *Klebsiella oxytoca*, and proteins for its secretion (65); flagellar proteins such as *Salmonella typhimurium* FlgH that forms the outer membrane L ring of the flagellar basal body (259); and LamB, which facilitates the uptake of maltose and maltodextrins across the bacterial outer membrane and acts as a general porin for small molecules (162). Many proteins in gram-positive bacteria have been suggested to be lipidated due to the presence of the consensus cleavage site (LAGC), and in some cases this has been confirmed by incorporation of radiolabelled fatty acid or interference by the antibiotic globomycin that inhibits processing by signal peptidase II (292). As in gram-negative bacteria, these lipoproteins are involved in diverse processes such as transport, adhesion, protein secretion, and conjugation. Examples include an *Alicyclobacillus* 40-kDa protein with similarity to enterobacterial maltose-binding proteins (122), and NisI, which affords *Lactococcus lactis* immunity to the antimicrobial peptide nisin (174).

(ii) N-myristoylated eukaryotic proteins. Another N-terminal lipidation is undergone by several eukaryotic proteins in which a myristoyl group is attached to the α-NH_2 group of the N-terminal glycine (position 2) following removal of the initiator methionine (140). The enzyme responsible, myristoyl-CoA:protein *N*-myristoyl transferase, is ubiquitous in eukary-

TABLE 4. Prokaryotic and eukaryotic proteins known or believed to possess internal amide-linked acyl groups

Protein	Total no. of amino acids	Acyl donor	Modified residue	Acyl group
Prokaryotic				
Pore-forming toxins				
E. coli HlyA	1,024	Acyl-ACP	K564	?[a]
			K690	?[a]
B. pertussis CyaA	1,706	Acyl-ACP?	K860[b]	C_{16}[b]
			K983	C_{14}[b], C_{16}
Eukaryotic				
Inflammatory cytokines				
TNF-α	233	Acyl-CoA	K19	C_{14}
			K20	C_{14}
IL-1α	271	Acyl-CoA	K82[c]	C_{14}
			K83[c]	C_{14}
Mitogenic receptors				
Insulin receptor (α and β)	1,382	?	?	C_{14}, C_{16}
Immunoglobulin				
μ_m	593	?	?	C_{14}
μ_s	572	?	?	C_{14}
Cholinergic receptors				
Nicotinic acetylcholine receptor				
α	457	?	?	C_{16}
β	501	?	?	C_{16}
Membrane complexes				
cytochrome *c* oxidase (subunit 1)	557	Acyl-ACP?	K324	C_{14}

[a] C_{14} preferred substrate in vitro.
[b] Acylated in *E. coli* only.
[c] Monoacylated preferred product.

otic cells. It was first isolated from *Saccharomyces cerevisiae* and has been extensively investigated (140, 302, 303, 332). It is almost exclusively specific for myristic acid and has stringent sequence requirements with respect to the 5 aa immediately distal to the glycine residue (140). An example of a myristoyl-CoA:protein *N*-myristoyl transferase using an acyl substrate other than myristoyl-CoA has been characterized in the retina (141, 163). Protein myristoylation is cotranslational and constitutive, and the product is usually a stably modified protein, although lipid removal from a mature protein has been reported (196).

N-myristoylation has been reported for more than 100 eukaryotic cellular and viral proteins, including a number of proteins involved in cell signalling that are also palmitoylated. Nearly all G-protein α-subunits are modified by an N-terminal myristoyl group (215), as are all members of the Src family of NRTKs. Other protein tyrosine kinases, while not lipidated themselves, signal through members of the Ras family of monomeric ("small") G proteins that are lipidated (Fig. 5), e.g., the Arf family involved in vesicular transport (38). Other N-myristoylated proteins include MARCKS (myristoylated alanine-rich C kinase substrate) (296); human immunodeficiency virus type 1 matrix protein (333); the calcium-binding sensor of photoreceptor cells, recoverin (2); and the hisactophilins of *Dictyostelium discoideum* (111).

(iii) Internal amide-linked acylation. As we have shown above, protein acylation is generally divided into labile internal modifications and stable modifications at the N and C termini. The mechanism of the stable acylation of HlyA at internal lysines via amide bonds is unique to the HlyA toxin family; no other prokaryotic proteins are known to be modified in this way. However, there are eukaryotic proteins that possibly have similarities to the HlyA acylation mechanism, but there has been little detailed characterization since their identification in the 1980s. Internal acylated lysines have been defined in four eukaryotic proteins, while another three proteins may possess hydroxylamine-resistant acyl groups not present at the N terminus. None of the associated acyltransferase activities has been characterized (Table 4).

Three inflammatory cytokines, TNF-α, IL-1α, and IL-1β, possess myristoyl groups attached through amide bonds to internal lysines. They are translated as 26- and 31-kDa precursors and are subsequently processed to produce extracellularly active, C-terminal 17-kDa mature proteins (117). The myristoylated residues actually lie within their cleaved propieces, and so acylation is not connected to the activity of secreted cytokines. Full-length TNF-α is myristoylated at lysine residues K19 and K20, and IL-α precursor is myristoylated at K82 and K83. A similar sequence in the IL-1β propiece is myristoylated but at a lower efficiency (288, 289). Lysyl *N*-ε-NH_2-myristoyl transferases that recognize small peptides (14 aa) as substrate and myristoyl-CoA as an acyl donor presumably exist for both TNF-α and IL-α, but these have not yet been identified (288, 289). However, even from the preliminary characterizations of peptide substrate and acyl donor, the acyltransferases are clearly different from HlyC.

Nicotinic acetylcholine receptor and insulin receptor are integral membrane glycoproteins composed of five and two subunits, respectively (57, 62). The presence of covalently bound fatty acids on these receptors has been explored by metabolic labelling of cultured cell lines with [^{3}H]myristic and [^{3}H]palmitic acids. The α- and β-subunits of both receptors

have an amide-linked fatty acid that is thought to be attached to internal free amino groups (119, 229). The site(s) of fatty acid attachment and the chemical nature of the lipid linkage have not been identified, but insensitivity to hydroxylamine indicates an amide bond, and the N-terminal residues are ruled out as sites of acylation since amino acid sequencing was not blocked. Membrane immunoglobulins are the recognition components of B-lymphocyte antigen receptors. Membrane immunoglobulin heavy chain (μ_m) reaches the cell surface and acts with light chain (L) as an antigen receptor. Following activation by antigen and the appropriate lymphokines and the synthesis of J chains, μ_m is secreted as a covalent pentamer $[(\mu_s)_2L_2]_5J$. Metabolic labelling with [^{3}H]myristic acid revealed that μ_m, μ_s, and light chains are covalently acylated probably by amide linkage to a lysine side chain since the myristate moiety is resistant to hydroxylamine (242).

Cytochrome *c* oxidase is a multisubunit enzyme complex of the inner mitochondrial membrane, catalyzing the terminal electron transfer and proton translocation steps of the mitochondrial electron transport system (6). Subunit 1 of cytochrome *c* oxidase of *Neurospora crassa* cells is myristoylated through an amide linkage at a lysine residue (K324) within one of its transmembrane domains (309).

From this section, one can see that there are many examples of processing leading to the addition of lipid groups to proteins in both prokaryotes and eukaryotes but that there are no prokaryotic mechanisms analogous to toxin maturation and only the possibility of a related mechanism in eukaryotes. The next section attempts to set out what is known about the roles of these different lipidations in biological function and how they are affected by other features possessed by the lipidated proteins; later, we will relate these to the effects produced by HlyA.

ROLES OF LIPIDATION IN PROTEIN FUNCTION

Increasing Affinity of Proteins for Biological Membranes

The increase in hydrophobicity resulting from the attachment of a fatty acid chain increases the association of many proteins with membranes. In gram-negative bacteria, N-terminal acylation localizes lipoproteins to the outer membrane, e.g., FlgH involved in membrane anchoring of the flagellar basal body, and PulS, promoting the correct localization of other membrane proteins involved in type III secretion of pullulanase (65, 259). In gram-positive organisms, many surface proteins are anchored by their C termini, but N-terminal lipidation may offer an additional membrane anchor generating a topology similar to that for Braun's lipoprotein (292). A similar function can be attributed to the acyl group of N-myristoylated proteins, many of which are cytosolic when unmyristoylated but acquire biological activity when bound to membranes through simple insertion of their myristate chain into the hydrophobic interior of the lipid bilayer (40, 140, 163, 265, 277, 296). Many thiopalmitoylated proteins are either integral membrane proteins or additionally N-myristoylated and do not require palmitoyl groups for membrane association. An exception appears to be the Gα-interacting protein (72). Membrane interaction is also promoted by prenylation, e.g., **a**-factor (53), G-protein γ subunits (217), and RTKs (64, 234, 290). Many eukaryotic cell surface proteins are anchored to the membrane by GPI, which in terms of stability is comparable to a hydrophobic peptide domain. If the GPI anchor is not attached, the protein is not localized on the cell surface but is instead retained in the cell (98).

It is easy to assume that the addition of a fatty acid with a long hydrophobic chain would be sufficient to convert a protein into a membrane-associated form, but the thermodynamics are not so unequivocal. It has been calculated from experiments with acylated peptides mixed with phospholipid vesicles that binding energy increases by 0.8 kcal/mol per -CH_2 group along an acyl chain (237, 266). The hydrophobic energy supplied by myristate (C_{14}) may not therefore be sufficient to attach a protein firmly to a membrane. This has been confirmed experimentally, since myristoylated proteins are anchored at a membrane surface but not embedded within it (161), and even a palmitoyl (C_{16}) chain cannot force a hydrophilic peptide sequence into a hydrophobic environment. Therefore, while palmitoylation next to a transmembrane segment may increase the partitioning of this hydrophobic segment into the membrane, the sole presence of a fatty acid moiety may not be sufficient for membrane binding, especially in hydrophilic proteins (148). However, the effort invested in the incorporation of fatty acids suggests that there is a benefit, especially where the lipid donor is a minor component of the substrate pool. This advantage may be that the low hydrophobicity of single lipid groups allows a reversible association with membranes (302).

Where single lipophilic groups determine low membrane binding, a simple means of increasing the membrane affinity of a protein is to incorporate a second fatty acid. The incorporation of fluorescently labelled peptides bearing double-lipid modifications (C_{20} + C_{20}, C_{14} + C_{16}, C_{16} + C_{15}, and C_{16} + C_{16}) into phospholipid vesicles has been used to monitor the effect of double acylation on membrane affinity (266). Lipopeptides with "double-anchor" motifs do indeed have a greater membrane affinity than that of monoacylated or monoprenylated peptides (78). Surprisingly, peptides possessing palmitoyl (C_{16}) groups associate with lipid bilayers even more than do geranygeranylated (C_{20}) peptides, and double palmitoylation may be the most hydrophobic lipid modification currently known in intracellular proteins. This view is supported by the finding that a MARCKS derivative bearing two N-terminal palmitic acids in place of the wild-type single myristoyl moiety is no longer released from the membrane (265).

Many membrane-associated proteins use a double anchor. Among the intracellular proteins that are covalently modified with acyl or isoprenyl groups, a significant number carry more than one hydrophobic group. In some N-myristoylated proteins such as those of the Src family (e.g., Lck and Fyn but not Src and Blk) and α-subunits of heterotrimeric G proteins, membrane binding is reinforced with additional palmitoylation of nearby cysteine residues (209, 247, 272, 308, 317, 334). The isoprenoid group binds to membranes with about the same affinity as myristate (266), and so membrane-bound prenylated proteins are also commonly doubly acylated. For instance, H-Ras and N-Ras which are farnesylated on their C-terminal cysteine residue are also reversibly S-palmitoylated on one or more nearby cysteines (198) (Fig. 6). The combined hydrophobic interactions of the two lipid moieties are necessary to anchor these proteins to the plasma membrane, as shown by the inability of prenylated, nonpalmitoylated H-Ras mutants to bind to plasma membranes (323). Other double-lipid substitutions, including double geranylgeranyl and geranylgeranyl-farnesyl modifications, have been suggested to occur on mammalian Rab proteins and yeast Ypt (71, 99, 269).

The membrane affinity of single acyl chains is not only enhanced by additional fatty acids. Both myristoylated and prenylated proteins use electrostatic interaction with the negative phospholipid head groups in the lipid bilayer to increase and decrease association with the membrane (218). The myristoyl groups of human immunodeficiency virus type 1 matrix protein

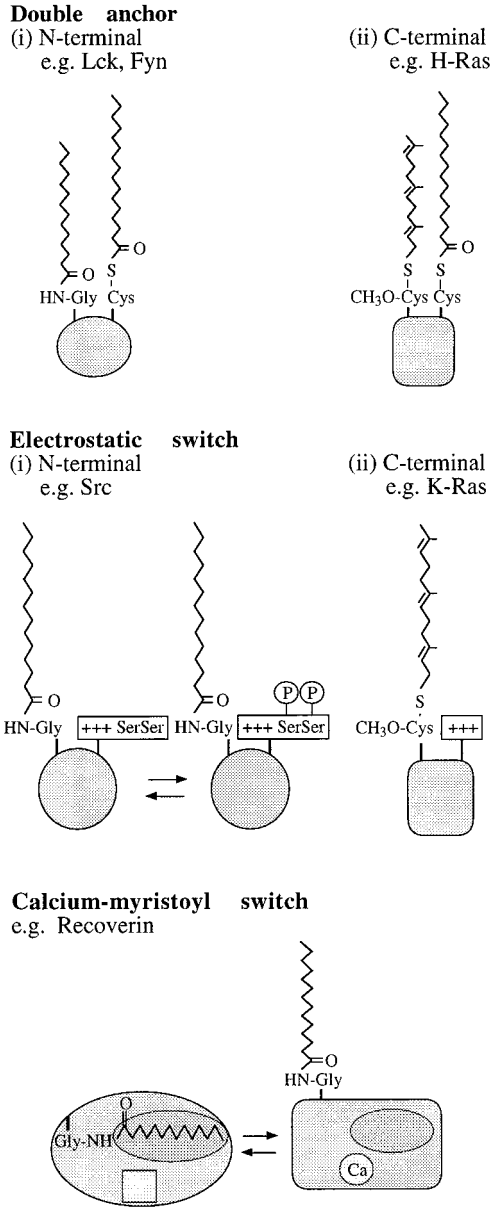

FIG. 6. Mechanisms to enhance the effects of single lipid groups. Double anchors increase hydrophobicity over single lipid groups. Members of the Src family of nonreceptor tyrosine kinases such as Lck or Fyn possess both an N-myristoylated glycine residue and a thioester-linked palmitoyl group. Small G-proteins such as H-Ras contain a C-terminal farnesyl group and a thioester-linked palmitoyl group. Electrostatic switches modulate the penetration of acyl chains into the membrane through the interaction of positively charged amino acids with the polar head groups of lipids. The myristoyl group of Src inserts into the bilayer when a basic domain interacts with acidic lipids, but phosphorylation of serine residues within this domain reduces the interaction. The hydrophobic interaction of the C-terminal farnesyl moiety of K-Ras with the bilayer is enhanced by an adjacent polybasic domain. The calcium-myristoyl switch of recoverin determines the accessibility of its lipid group. In the Ca^{2+}-free state, the myristoyl group occupies a hydrophobic cluster (shaded oval), while in the Ca^{2+}-bound state, the myristoyl group leaves the hydrophobic cluster and is exposed to interact with membranes or other proteins.

cooperate with basic residues to interact with the acidic phospholipids in the membrane (333), and similar cooperative interactions between a C-terminal farnesyl moiety and an adjacent hexalysine domain have been observed for K-Ras (198, 222) (Fig. 6). Further subtlety arises from this additional electrostatic interaction, since in some proteins the phosphorylation of serine residues within basic sequences reduces the attraction for acidic lipids, providing an "electrostatic switch" for reversible binding, e.g., MARCKS and Src (161, 247, 296). N-myristoylated hisactophilins possess an electrostatic binding site containing both basic and acidic residues, so that below pH 7 electrostatic attraction increases interaction with a lipid bilayer while above pH 7 it determines electrostatic repulsion (111). Other myristoyl switch mechanisms are responsible for the reversible membrane-binding of ADP-ribosylation factor and recoverin. ADP-ribosylation factor requires the binding of GTP first to expose an otherwise hidden myristoyl group and a patch of basic residues that then promote membrane insertion, while in the case of recoverin it is the binding of Ca^{2+} that exposes a myristoyl moiety masked within a hydrophobic groove, allowing it to interact with a lipid bilayer (2, 5, 38) (Fig. 6).

Enhancement of Protein-Protein Interactions

Lipid attached to a protein acts not only as a mediator of membrane association but also as a determinant of specific protein-protein interactions and the assembly of membrane protein complexes. One obvious reason why membrane binding aids the interaction between proteins is that it reduces diffusion from three to two dimensions and increases their effective concentration approximately 1,000-fold. It may also expedite the lateral movement of membrane proteins, which may be required for rapid cross-linking and oligomerization. Nevertheless, very little is known about how acyl groups alter protein structure, especially during the change from the monomeric non-membrane-associated protein to the multimeric membrane-associated protein. Signal transduction is one area in which the contact of proteins with other proteins is crucial, and acylation plays a central role in these processes. The palmitoylation of GPCRs, the myristoylation, palmitoylation and prenylation of heterotrimeric G-proteins, the prenylation of Ras-related small G-proteins, the myristoylation and palmitoylation of Src family tyrosine kinases, and the glypiation of GPI-linked membrane proteins are all critical at different stages of signal transduction for reasons other than influencing membrane affinity (110, 199, 222, 247, 317, 331) (Fig. 5).

Approximately 1,000 different GPCRs exist in mammals, each member interacting with a limited number of closely related G-protein heterotrimers, and palmitoylation may play a role in this interaction since it is necessary for coupling to β_2-adrenoceptors although it is not essential for other receptors (115, 139, 153). The proximity of sites for palmitoylation, G-protein binding, and RTK-mediated phosphorylation on GPCRs (321) allows an interplay such that the state of palmitoylation influences RTK recognition of the agonist-occupied receptor and plays a role in signal desensitization through the removal of the GPCR from the cell surface (213, 234). As well as associating with GPCRs, G-protein α-subunits interact with $\beta\gamma$-subunit complexes and with effector molecules such as adenylyl cyclase. The N-terminal myristoyl group on Gα subunits specifically contributes to G$\beta\gamma$ binding (19, 183, 317). There may not be direct contact between the fatty acid on α and the protein in $\beta\gamma$ (32), but the isoprenoid group attached to the γ polypeptide does contact Gα in the $\alpha\beta\gamma$ trimer (217, 317, 325, 331). In addition, nearly all Gα subunits are reversibly palmitoylated, especially when the α subunit is not complexed with $\beta\gamma$ (145, 317).

The role of the farnesylation of the *Saccharomyces* pheromone **a**-factor is not restricted to increasing hydrophobicity but may be important in binding the **a**-factor receptor (68). Lipi-

dation also affects the interaction of many small G proteins such as Rho and Rab with their regulatory proteins, e.g., GDP-dissociation inhibitors (GDIs) and GDI displacement factor (198). The prenyl groups of small G proteins may bind to pockets in GDIs to form soluble complexes (150, 157, 324).

GPI-anchored proteins are cell surface proteins that are involved in signal transduction; e.g., GPI-anchored glycoproteins of *Trypanosoma cruzi* induce proinflammatory cytokine synthesis by macrophages (49). To generate intracellular signals, GPI-anchored proteins such as Thy-1 that do not extend into the cytoplasm must interact with proteins on the inner face of the membrane such as Gα subunits and the Src family of nonreceptor tyrosine kinases. These interactions are influenced by the presence of saturated acyl chains; e.g., Src family members need to be N myristoylated and palmitoylated to interact with GPI-linked proteins (271), but this influence may be nonspecific and caused by concentration of lipidated proteins within detergent-resistant membrane domains of eukaryotic cells such as caveolae (41, 276). Dual palmitoylation may have additional effects more subtle than an increase in membrane affinity such as preferential targeting to the plasma membrane or Golgi apparatus (33, 149). Recognition between Src kinases and their substrates is also affected by their state of palmitoylation (255).

INTERNAL AMIDE-LINKED ACYLATION AND HlyA TOXIN FUNCTION

Despite the differences in the structure of the lipid groups attached at or near the N and C termini of proteins, as we have shown above, their consequences can be discussed together in terms of affecting membrane affinity and/or protein-protein interactions. These functions are also applicable to the modification of internal lysine residues with long-chain fatty acids. Since the *E. coli* HlyA fatty acylation appears to be most similar to the internal amide-linked acylation found on a small number of eukaryotic proteins, we now discuss separately the consequences of acylation proposed specifically for these proteins.

Eukaryotic Internal Amide-Linked Acylation

Lipopeptides with long-chain fatty acids attached to the side group of lysine residues have been used to investigate the biological effect of the unusual lipidation involving internal amide-linked acylation. A 14-aa hydrophilic peptide with a palmitoylated lysine at either the N- or C-terminal end is able to enter intact human cells, whereas the unmodified peptide is not (185). This system used synthetically modified peptides, but biological peptides do contain internal N^{ε}-palmitoyl-lysine residues. An isoform of the 35-residue surfactant protein SP-C possesses two thioester-linked palmitoylated cysteine residues as well as a palmitoyl group linked to the ε-amino group of Lys11. The origin of this third modification appears to be nonenzymatic, and its biological significance is unclear (105). The acylation of phospholipase A_2 at the ε-amino groups of lysine residues converts the soluble enzyme into a membrane-penetrating form with an increased interaction with phospholipid monolayers (270). While this modification followed chemical treatment with short (C_8) acyl chains and may not be physiologically relevant, the findings nevertheless appear relevant to the other eukaryotic proteins (e.g., TNF-α, IL-1, and cytochrome *c* oxidase) known or suggested to carry internal amide-linked fatty acids (Table 4).

The acylated lysine residues of TNF-α are located immediately downstream from a hydrophobic, membrane-spanning segment of the propiece, and so acylation may facilitate the membrane localization or insertion of the precursor molecule by enhancing overall hydrophobicity. Such a role for the acylation of cytokine propieces is supported by the fact that both the TNF-α and IL-α precursor proteins are active as plasma membrane-associated proteins whereas the poorly myristoylated IL-1β precursor is active only as the processed secreted form (25, 70). Dansylated myristoyl acceptor peptides derived from IL-α have demonstrated the specific binding of the myristoylated form to the inner leaflet of erythrocyte ghosts, suggesting that acylation does indeed facilitate membrane binding (289). The unmodified subunit 1 of cytochrome *c* oxidase is already extremely hydrophobic, with several predicted transmembrane α-helices, and so it seems unlikely that acylation is required for integration of the protein into the lipid bilayer of the inner mitochondrial membrane. It is possible that myristoylation of subunit 1 encourages protein-protein interactions, contributing to assembly with subunit 2 and perhaps other subunits of this enzyme complex (309). The role of covalently bound fatty acids in the insulin receptor is unclear, but they may have important implications in terms of receptor stability, life cycle, or function (119). Immunoglobulin heavy chain (μ_m) is initially synthesized as a relatively hydrophilic protein, which is nonetheless stably anchored in the endoplasmic reticulum membrane. Myristoylation of μ_m correlates with its transport to the cell surface and its conversion to a relatively hydrophobic form. However, myristoylation does not result in detergent solubility, suggesting that its effect on μ_m may be an indirect one through mediating intermolecular interactions during its assembly and transport to the cell surface (242). Similarly, acylation of the nicotinic acetylcholine receptor, an integral membrane protein, appears to have a greater influence on protein-protein interaction rather than membrane affinity. These interactions may not be restricted to the receptor subunits but may include molecular chaperones such as calnexin, which forms complexes with nascent α subunits (96). Subunit assembly of nicotinic acetylcholine receptor is relatively slow and begins with the interaction of α and δ subunits in the endoplasmic reticulum to form a heterodimer. This process requires the tethering of the α subunit to the membrane, but whether this is provided by a lipid group is not known (316). Nevertheless, in the absence of acylation, subunit assembly is impaired, possibly because nonacylated subunits are degraded before they can be transported to the plasma membrane (207).

The roles of acylation at internal lysine residues thus appear to be the same as those of other lipid modifications. Eukaryotic proteins with internal amide-linked acyl groups form active membrane-associated complexes. Can this be linked to HlyA toxin function in eukaryotic membranes? Is it a coincidence that six of the seven eukaryotic proteins with internal amide-linked acyl groups are involved in signalling events either as cytokines or as receptors?

Host Cell Targeting by the HlyA Toxin Family

We have set out how fatty acylation of proteins can be central to anchoring to membranes, signal transduction, and oligomerization. The functions of the HlyA toxin suggest that its acylation could contribute in all three of these areas. It is tempting to try and explain the differences in the cell type and host species attacked by the toxins of the HlyA family in terms of what might be differences in their posttranslational modification. One interpretation of domain exchanges between hemolysins and leukotoxins and between leukotoxins that attack cells from different species could be that separate sequences account for erythrocyte specificity (~aa 450 to 700 on HlyA)

and leukocyte specificity (~aa 700 to 850) (88, 175, 202). Although these regions approximate to the acylation sites of HlyA, examination of KI and KII within the toxin family does not allow a simple view that their acylation might individually determine erythrocyte and leukocyte specificity (Table 3). For instance, ApxIIA and LktA are cytolytic but do not possess KII, while AaltA and LktA are not hemolytic but do possess KI. The number of acylation sites also appears not to be decisive, since ApxIIA, a hemolysin, and LktA, a leukotoxin, both have one site whereas HlyA, a hemolysin, and AaltA, a leukotoxin, both have two sites. More subtle models are also not substantiated experimentally. Of the two (KI and KII) acylated lysines responsible for the hemolytic activity of HlyA, only KI is conserved in the strictly leukotoxic LktA (282). A simple hypothesis would be that the acylation of KI determines targeting to leukocytes while acylation at both KI and KII is required for erythrolysis. Although simple, this hypothesis has proved wrong, since HlyA with a substituted KII lost not only hemolytic activity but also its leukotoxic activity against human polymorphonuclear leukocytes (31, 239), and an N684K substitution, placing a lysine residue at the prospective KII site of LktA, failed to confer activity against erythrocytes (239). As previously indicated by studies with hybrid gene constructions (88, 175, 202), the structural basis of target cell specificity is unlikely to lie in single features of the toxins. In addition, the contribution made by putative receptors is unknown. Identification of all the acylation sites of the family of toxins will not be sufficient to explain cell targeting but will provide a further understanding of the mode of action of individual toxins at the molecular level.

Toxin Association with Eukaryotic Cell Membranes

A discussion of cell targeting in terms of toxin acylation may be more revealing if one considers the separate processes of membrane insertion, host cell signalling, and pore formation. The influence of fatty acylation on HlyA membrane binding has been investigated, but the results are contradictory. Several research groups have concluded that unmodified, inactive pro-HlyA is unable to bind to erythrocyte membranes (37, 187) while the acylation of CyaA has been proposed to be essential for tight association with target cells (109). However, other work has suggested that acylation does not increase the affinity of toxin either to artificial liposomes or to real mammalian target membranes (15, 76, 210, 275). An explanation of these apparently opposing conclusions may lie in small differences in the preparation of the toxin samples. Fully modified toxin preparations lose their activity after dilution, possibly through self-aggregation and an inability to bind, and it appears that this tendency may be greater for unacylated than for acylated toxin and for calcium-bound rather than calcium-free toxin (11, 285). In addition, toxin at least partially denatured by the action of chaotropic agents appears to acquire the potential to bind lipid bilayers in an acylation-dependent manner (285). Under certain conditions, HlyA binding to erythrocytes is effective in the absence of acylation, Ca^{2+} binding, and the N-terminal hydrophobic region (15); there may therefore be no single factor that is decisive for in vivo membrane insertion. Whether the primary interaction and binding of HlyA to target membranes is dependent on fatty acylation is therefore unresolved and will probably require a more sophisticated approach than that allowed by in vivo systems.

Theoretically, by increasing the hydrophobicity of HlyA, the influence of fatty acylation would be to increase membrane binding. However, an alternative view is that the N-terminal hydrophobic domain of HlyA, possessing eight transmembrane helices, is sufficient for at least initial membrane binding and that before insertion is complete, the acyl chains are separated from the aqueous environment, perhaps hidden within hydrophobic tunnels. The transition from a membrane-bound to a membrane-inserted state could involve a conformational change that may at least partially release the acyl chains to the lipid bilayer. This transition could lie behind the temperature sensitivity of the lytic process; binding of HlyA to a membrane is slower at 4°C than at 37°C, but the amount eventually bound is constant. However, at the lower temperature, no cell lysis results despite the binding (15, 76, 210).

An essential step in toxin action, in addition to acylation, is the binding of calcium at the glycine-rich repeats of the toxin. Here the "Ca^{2+}-myristoyl switch" of recoverin may be useful as a functional model (Fig. 6) (2), with the binding of Ca^{2+} being required to expose the acyl groups and make them available to the lipid bilayer. Certainly, Ca^{2+} binding by HlyA does appear to increase its overall surface hydrophobicity (11). It is possible that the binding of calcium affects the conformation of the toxin not only at KII adjacent to the glycine-rich repeats but also at KI more than 170 aa away, since in the presence of Ca^{2+} both KI and KII are no longer recognized by HlyC in vitro (283). While a large conformational change has not been detected by circular dichroism for HlyA on calcium binding, it has been shown for CyaA (11, 250). Why should the doubly acylated HlyA require an additional mechanism to increase membrane affinity when double acylations are among the strongest modifications known? The answer may lie in the HlyA acylation sites being 126 aa apart whereas in doubly modified N-myristoylated and prenylated proteins the two lipid groups are close together. It is more correct to view HlyA not as a doubly anchored protein but as being monoacylated in two places, a view corroborated by the existence of a sole palmitoyl group on native CyaA. The highly charged nature of the residues either side of KI and KII (30% of the flanking 20 residues are DEKR, with no overall positive charge) further increases the instability of any potential membrane association. The acylated lysines of HlyA do not have neighboring clusters of hydrophobic or positively charged residues as for K-Ras whether considered as a linear sequence, a helix, or a β-sheet (Fig. 3). Without these, single myristic or even palmitic side chains are unlikely to provide sufficient hydrophobic interaction to stabilize a transmembrane region even if there are additional contributions by the lysine side groups. Rather, the acyl groups on HlyA may act at most as anchorage points to the surface of the lipid bilayer.

Effects of HlyA Toxin on Host Cell Signalling and Cytokine Production

The hydrophobicity of fatty acylation does not appear to be a simple key to HlyA targeting to mammalian cell membranes, but it may be significant in mediating specific protein-protein and/or protein-lipid interactions. Investigations into the effect of acylation on the ability of sublytic concentrations of HlyA to stimulate a host cell response have been complicated, perhaps significantly, by the presence of LPS in toxin preparations. At least the major component of HlyA binding to granulocytes appears to be non-receptor mediated, with subsequent triggering of the respiratory burst presumably not through the occupancy of a receptor but through a short-circuiting of the classical signal transduction pathway (103, 165). Acylation may therefore enhance protein-protein interactions between HlyA and components of a signal transduction pathway, perhaps by targeting to detergent-resistant domains (Fig. 5). With acylation sites C-terminal to a multiple transmembrane helical do-

main, HlyA is at least superficially similar to the GPCRs, but unlike the labile thioester-linked palmitoylation of GPCRs, the amide-linked modification of HlyA would not be reversible. Effects of acyl groups on cytokine induction, however, are not restricted to a model incorporating complex protein-protein interactions. A synthetic lipopeptide of the N terminus of a bacterial lipoprotein stimulates the synthesis of IL-1, IL-6, and TNF-α by murine macrophages (114). Lipoteichoic acids from *Streptococcus faecalis* stimulate murine mononuclear cells to release both IL-1 and TNF, but deacylation stops the cytokine-stimulating activity (304), and deacylation of lipoarabinomannan of *Mycobacterium* spp. almost totally inhibits its capacity to induce cytokine synthesis (14). Intriguingly, another response of monocytes exposed to lipoteichoic acid is to increase the expression of CD11a/CD18, a putative receptor that may be recognized by RTX toxins (51, 176).

HlyA Pore Formation in Host Cell Membranes

As well as being a pseudo-chemokine, HlyA is cytotoxic as a result of its ability to form pores. If monomers of the toxin must combine within the membrane to form pores, perhaps the acyl groups are involved in protein-protein interactions to bring about oligomerization. By providing anchorage points in the membrane, acyl groups may prevent essential domains from looping away from the membrane surface and may enhance lateral diffusion to accelerate the contact between them. The assembly of the acylated subunit 1 into the cytochrome oxidase complex and the interaction of the acyl groups involved in contact between the α and γ subunits of heterotrimeric G-proteins may provide useful analogies to this process (Fig. 5; Table 4). The most dramatic effect of the absence of fatty acylation on the action of HlyA indeed appears to be at the pore-forming step. Both acyl modifications must be present for HlyA to form channels. Loss of either acyl group results in the virtual abolition (>99.5%) of cytolytic activity (282). This is at least true for erythrocytes and human polymorphonuclear leukocytes. Monoacylated HlyA derivatives lacking either KI or KII do retain a reduced ability to form pores in bovine lymphocytes (BL-3 cells) (239). Why pore formation in BL-3 cells should be less dependent on toxin acylation is unknown, but the impression of systems involving bovine lymphocytes and the specific *P. haemolytica* leukotoxin LktA is that they represent a special case.

An influence of acylation on pore formation has also been demonstrated for CyaA. After expression in *E. coli*, CyaA carries an acyl group on K860 in addition to the K983 acylated in the native host. This overacylated toxin is impaired in the formation of the CyaA channels, being about 20% as hemolytically active as the wild-type CyaA (109). The hemolytic activity of HlyA absolutely requires double acylation, while monoacylation is sufficient for the hemolytic activity of CyaA. However, while both HlyA and CyaA are described as hemolysins, there is a dramatic difference in their erythrolytic activity, such that CyaA hemolytic assays are performed at nanomolar toxin concentrations but HlyA assays require only picomolar toxin concentrations. Monoacylated HlyA derivatives retaining only <0.5% of the wild-type activity are correctly defined as nonhemolytic but are still more hemolytic than the wild-type CyaA toxin. Nevertheless, whereas acylation at KI is essential for pore formation by HlyA, acylation at KI actually seems to impede the hemolytic activity of CyaA. The primary sequences 50 aa either side of KI are only 30% identical between HlyA and CyaA (283), so perhaps a reason for this difference will be confined to individual structures. It might be significant that KI is the acylation site closest to the toxin hydrophobic regions, with the hydrophobic region of CyaA possibly having an intrinsically stable topology while that of HlyA requires its C-terminal side to be stabilized by an anchorage to the membrane (Fig. 2). Evidence that the acyl chains attached to HlyA may provide membrane anchors and that their stability in the membrane is influential in pore formation is provided by the observation that doubly palmitoylated HlyA has a specific hemolytic activity at least fivefold higher than that of doubly myristoylated HlyA (285).

PERSPECTIVE

The pore-forming HlyA of *E. coli* represents a remarkable class of bacterial toxins that requires a posttranslational modification for activity. The maturation of HlyA by the covalent amide linkage of fatty acids to internal lysine residues is determined by an apparently unique, virulence-related lysyl-acyltransferase activity unlike any other in prokaryotes. Analogous activities have been identified in a few eukaryotic systems, but these have not been isolated. The mechanism underlying this activity may have implications for the value of acyltransferases as therapeutic targets, as realized for the enzymes in the LPS biosynthetic pathway (230) and the farnesyltransferase of oncogenic Ras (263).

Predictions can be made about the possible roles that the HlyA acylation plays in endowing biological activity. One general consequence of lipidation is an increase in the membrane affinity of a protein, but this may not be relevant to HlyA. Protein lipidation is also important in the regulation of protein-protein interactions. High concentrations of HlyA produce cell lysis, possibly through the formation of oligomeric pores, a process that is absolutely dependent on acylation. The association of acylated eukaryotic proteins into active membrane-bound complexes, as exemplified by internally acylated cytochrome oxidase or the heterotrimeric G-proteins, may provide useful analogies for the design of a model for these bacterial toxins.

In eukaryotic cells, mitogenic receptor activation is linked through tyrosine and serine/threonine kinase pathways to the activation of phospholipase C and the liberation of diacylglycerol (199, 264). Sublytic concentrations of HlyA also trigger the generation of diacylglycerol (28, 30), but where it interacts with the host signalling pathway is unknown. It may be significant that both membrane immunoglobulin and insulin receptors appear to possess identical amide-linked, internal fatty acylation, as found on HlyA. In addition, the Ras and Src components of these signalling pathways, and the heterotrimeric G-proteins and their coupled receptors, all have acylated residues. A role for the HlyA acyl groups in signalling would not necessarily require them to cross into the inner lipid layer of the eukaryotic membrane, since GPI-anchored proteins remain on the outside of the cell and are believed to signal through Lck and Fyn NRTKs (271). However, the delivery of hydrophilic peptides containing N^{ε}-palmitoyl-lysine residues into the cytoplasm of intact cells suggests that the cell membrane may not be an impervious barrier to the acylated domains of HlyA (185). HlyA also affects cytokine production, such as stimulating the release of IL-1β and TNF-α from human monocytes. It has been suggested that virulent *E. coli* has receptors for IL-1 and responds to its binding by proliferation (244), so the HlyA-mediated release of IL-1 might act as an environmental signal. In addition, it is known that IL-1β increases the cytotoxicity of Shiga toxin toward human vascular epithelial cells by increasing the synthesis of its receptor, globotriaosylceramide (154). There is therefore the possibility of a similar link between the action of HlyA and Shiga toxin in *E.*

coli pathogenesis. A relationship between the acylation of HlyA and the acylation of lysine residues in the precursor, membrane-bound forms of IL-1 and TNF-α would support speculation that cytokine-inducing proteins produced by bacteria are evolutionary precursors of mammalian cytokines (120). If the posttranslational acylation of HlyA does indeed allow it to mimic a eukaryotic cell counterpart, it will be a further example of interaction between prokaryotic and eukaryotic cells.

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